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Exploring the roles of inputs to hippocampal area CA1

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Abstract

Place cells in the hippocampus fire in specific locations within an environment. The aim of this thesis is to investigate the different inputs to the hippocampus and what they contribute to place cell activity and performance of hippocampus-dependent tasks. Place cell activity can also be modulated by relevant features of a task such as a future destination or trajectory. Initial experiments investigated the origin and function of this trajectory-dependent activity and later experiments targeted the medial entorhinal cortex inputs to the hippocampal formation and investigated what they contributed to place cell activity and behaviour.

The purpose of the first study was to determine whether trajectory dependent activity occurs in CA3 in a hippocampus-dependent serial-reversal task on the double-Y-maze and to compare it with that seen in CA1. Place cells in both CA3 and CA1 were recorded in rats trained on a serial-reversal task on a double-Y-maze. Rats were trained to run from a start box through two Y-junctions to one of four goal locations. After 10 trials the reward was moved to a new location, until all the boxes had been rewarded. Previous research has found that 44% of CA1 place cells with fields in the start areas of the maze show trajectory-dependent activity in rats trained on the task. This study found that a similar proportion of CA3 place cells also show trajectory-dependent activity in rats trained on this task and that this activity develops at the same time point as the task is learned. This result suggests that trajectory-dependent activity may be generated earlier in the circuit than CA1.

Secondly, the contribution of the nucleus reuniens (N.Re) to spatial tasks was investigated. Previously, trajectory-dependent activity has been found to reach the hippocampus via N.Re, however this was shown in a hippocampus-independent task. To investigate the possible role that this input may play in behaviour, N.Re was lesioned and animals were tested on acquisition and performance of the double-Y-maze serial-reversal task described previously. Surprisingly, lesions had no effects on either learning or performance. Taken together with previous data from other studies, this suggests that trajectory dependent activity is not one unique phenomenon but is rather multiple similar phenomena which may originate in different brain regions and

fulfil different roles in navigation depending on the demands of the task. In addition, animals were tested on tasks involving allocentric or egocentric navigation. Results suggest that N.Re may have a role in the selection or performance of allocentric navigation but not egocentric navigation.

Thirdly, the role of inputs from the medial entorhinal cortex (MEC) to place cells was investigated. Consistent with previous research, MEC lesions resulted in larger, less precise place fields in CA1 place cells. By performing cue-rotation experiments using either distal or proximal cues it was observed that place fields in the MEC lesion animals were not anchored to distal cues but were either stable or anchored to other aspects of the environment. However, place cells in the MEC lesion group still followed proximal cues suggesting that the deficit is restricted to distal landmarks. This suggests that the MEC may process distal landmark information allowing the use of distal landmarks for orientation and self-location within an environment.

This thesis contributes a better understanding of the role and origins of trajectory dependent activity as well as a novel finding that the MEC contributes information about distal landmarks to the hippocampus.

Lay Summary

The hippocampus is a key brain area involved in navigation and memory. Neurons in the hippocampus known as 'place cells' become active only when you are in a specific place and so can tell you where you are within any environment. This thesis is investigating different brain areas which send information to place cells and is exploring the different types of spatial information which they may be contributing.

Place cells can represent more than just current location. Specifically they can represent your current location plus where you are coming from or going to. This thesis investigates whether this representation of trajectory is seen in an earlier area within the hippocampal circuit. The same amount of trajectory-representation was observed in the new area, suggesting that this representation may be generated earlier in the circuit.

This representation of trajectory may be necessary to learn or solve trajectory-based navigation tasks. After removing the nucleus reuniens (a brain area which is thought to contribute trajectory information to place cells) I tested whether navigation was impaired in several different tasks. Surprisingly, navigation was not impaired in the task in which trajectory-representation is seen in the hippocampus. This suggests that other inputs may compensate either by contributing other trajectory information or by representing the task in a different way. The data obtained also suggest that the nucleus reuniens is involved in certain types of navigational strategy but not others. Allocentric navigation refers to navigation based on a mental map of a place in relation to landmarks within an environment (for example: "go north to get to the destination"), while egocentric navigation is based on learning a sequence of right/left turns and distances (for example "turn right to get to the destination"). Rats without the nucleus reuniens were better at egocentric strategies and found it harder to learn a purely allocentric task, suggesting that the nucleus reuniens is involved in allocentric strategy selection.

Finally I looked at the contribution of the medial entorhinal cortex (MEC), which is a major spatial input to the hippocampus. Removing the MEC changed the types of landmarks that place cells used to orient within an environment. Rather than orienting relative to distant landmarks (similar to windows or pictures on the wall of a room), place cells oriented relative to local landmarks (similar to objects within the room). This suggests that the MEC inputs allow mapping of environments relative to far away landmarks but not close landmarks.

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Declaration

I declare that I composed this thesis myself. All work is my own except where indicated in the text and this work has not been submitted for any other degree or professional qualification.

Elizabeth Allison

Recording and initial clustering analysis for one cohort of rats used in Chapter 2 was performed with the help of Emily Cox who volunteered in the lab to learn single-unit recording. The behavioural training in Chapter 3 for the double-Y-maze serial reversal task, delayed alternation task, and reference memory water maze task was performed by Thomas Ripard and Georgy Yukhnovich; all surgeries, data analysis, and other behavioural training was performed by the author. Recording, initial clustering analysis of the first cohort of animals in Chapter 4 as well as initial experiment protocol development were performed with the help of Julia Thomas who worked on the experiment as part of her fourth year undergraduate project.

Primary Matlab scripts for data extraction were written by Sturla Molden (Centre for the Biology of Memory, NTNU), secondary Matlab scripts for processing and running clustering algorithms were written by Dr Steven Huang (CCNS, Edinburgh), final analysis and visualization scripts were written by the author or modified from combinations of previous scripts by Dr Steven Huang and Dr Roderick Grieves.

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1 Abbreviations

ANOVA	Analysis of variance
AP	Anterior-Posterior axis
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
DNMTP	Delayed non-matching to position
DV	Dorso-ventral axis
EC	Entorhinal cortex
ID	Isolation distance
LEC	Lateral entorhinal cortex
LFP	Local field potential
LII	Layer II of the MEC
LII	Layer III of the MEC
MEC	Medial entorhinal cortex
ML	Medio-lateral axis
mPFC	Medial prefrontal cortex
NMDARs	N-methyl-D-aspartate receptor
N.Re	Nucleus Reuniens
N.Rh	Rhomboid Nucleus
RAM	Radial arm maze
RC-DNM	Repeated choice delayed non-matching
RM-ANOVA	Repeated measures ANOVA
SEM	Standard error of the mean
TPI	Threads per inch
VC-DNM	Varying choice delayed non-matching
VTE	Vicarious trial and error
5CSRT	Five choice serial reaction time
~	Approximately

1 Introduction

1.1 The role of hippocampal place cells in navigation

1.1.1 The history of the hippocampus and navigation

The hippocampus has been studied as a memory centre since the famous case of Patient H.M. (Scoville and Milner, 1957). H.M. had both his hippocampi removed as treatment for epilepsy, with the unexpected side-effect that he lost the ability to form new episodic memories, but his procedural memory was unaffected (Corkin 1965). Although it is likely that the deficits H.M. suffered were partially related to loss of surrounding tissue as well as hippocampus, this case kick-started the study of the hippocampus and memory. After O'Keefe and Dostrovsky (1971) discovered place cells in the hippocampus, research shifted to exploring the role of the hippocampus in navigation. O'Keefe, with Nadel, went on to propose that the hippocampus supported a cognitive map (1978). The cognitive map theory suggests that there is an internal map of the environment that allows the representation of different locations and their relative positions in a meaningful way (Tolman 1948). Based on the spatial firing properties of hippocampal neurons as well as data from lesion experiments, O'Keefe and Nadel suggested that the hippocampus could support such a map. The idea that the hippocampus is important for spatial memory and navigation has been largely accepted ever since and has been backed up by many behavioural experiments. For example, total hippocampal lesions in rats were found to cause permanent impairments in navigating to a place unmarked by any local cues in a watermaze (Morris *et al.* 1982). Another human case study, patient E.P. who also had bilateral hippocampal damage, was unable to learn new maps although he could remember the layout of his neighbourhood before the damage (Teng and Squire, 1999). In addition, H.M. also showed some spatial impairments, although he did learn to navigate around new environments (Corkin 2002). fMRI data from healthy people showed an increase in activity in the hippocampus during a complex virtual-navigational task (Maguire *et al.* 1996, 1997). Electrophysiological recordings from epilepsy patients navigating in virtual reality have also found evidence of potential place cells (Ekstrom *et al.* 2003),

supporting the idea that in both rats and humans the hippocampus may support a cognitive map of the environment.

Navigation can be performed in several different ways. Egocentric navigation is used to describe navigation relative to oneself using a learned sequence of actions (e.g. turn right, travel 10m then turn left). Allocentric navigation refers to navigation using a mental map of an environment. Places can be represented in relation to multiple landmarks allowing flexible navigation between locations. The role of the hippocampus in navigation appears to be restricted to allocentric navigation (Packard and McGaugh. 1996) or complex egocentric navigation involving multiple turns (Rondi-Reig *et al.* 2006, Fouquet *et al.* 2010), presumably because alternative striatal circuits can support simple egocentric response tasks (Packard and McGaugh, 1996).

1.1.2 Place cells

Place cells were first observed by O'Keefe and Dostrovsky in 1971. They recorded from the rat dorsal hippocampus from CA1 and dentate gyrus and found neurons that fired only when the rat was in a certain location of the testing platform. By changing or moving the various types of cues available to the animal, they also showed that these 'place cells' were not responding to one specific cue in the environment but seemed to respond to the combinations of cues which made up being in that location. Cue changes large enough to change the cells' firing patterns caused the animals to respond as if it was a novel environment, suggesting that the cell was encoding the location itself rather than the visual or olfactory cues alone. O'Keefe defined a place cell as "a cell which constructs the notion of a place in an environment by connecting together several multisensory inputs each of which can be perceived when the animal is in a particular part of an environment" (O'Keefe, 1979). Place cells have since been recorded in mice (McHugh *et al.* 1996), monkeys (Matsumura *et al.* 1999), humans (Ekstrom *et al.* 2003), and recently 3D place cells were even recorded from flying bats (Yartsev *et al.* 2013). Similar activity has also been recorded from "spatial view cells" in the hippocampus of primates and humans which codes for the place being viewed, rather than current physical location (Rolls *et al.* 1997, Ekstrom *et al.* 2003). Figure 1 illustrates how place cells are usually recorded. A rat is placed in an environment and

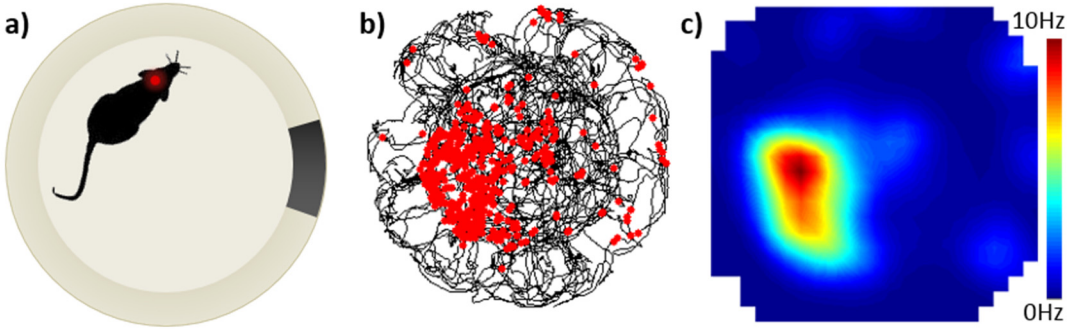


Figure 1: Illustration of place cell recording. a) pyramidal neuron spikes are recorded as a rat explores a cylinder, b) the animal's trajectory over the session (grey) with superimposed spikes from one neuron recorded by the author(red) c) a firing rate map of the environment

hippocampal pyramidal neuron spikes are recorded, along with the animal's position based on a light on its head (Figure 1a). This position information can then be used to create a map of the animal's path through the environment on which the spikes can be superimposed (Figure 1b), or a firing rate map which shows the neuron's average activity in each part of the environment (Figure 1c).

By recording from multiple cells at once, it was shown that different place cells fired in different locations or 'place fields' within the environment, and that collectively, they could represent the whole environment. In a novel environment, cells developed new fields to form a new map independent of the previous one (Wilson and McNaughton 1993) a phenomenon known as "remapping". It was also found by recording multiple cells at once, that the representation of space was not topographical; place cells with adjacent place fields were not necessarily adjacent in the brain and vice versa (O'Keefe *et al.* 1998, Redish *et al.* 2001). In a novel environment, or when the environment was radically changed, place cells would remap (O'Keefe and Conway, 1978, Muller and Kubie, 1987). Remapping (or more specifically 'global remapping') refers to the response of place cells to major changes to an environment; forming a place field in a new location or ceasing to fire entirely (Figure 2a). Subtler changes to contextual cues can lead to changes in firing rate within the place field without a change in location, which is known as rate-remapping (Leutgeb *et al.* 2005c) (Figure 2b). In addition, place cells may respond to changes in the environment in a non-coherent way with cells showing different responses to changes in the

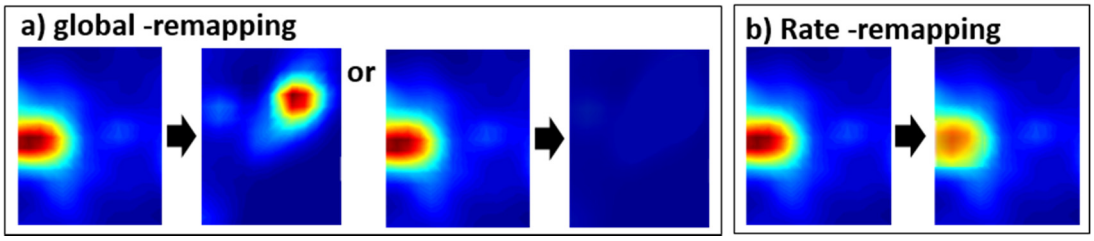


Figure 2: Illustration of the types of place field remapping: a) illustration of global remapping: place fields either change location between recording sessions or disappear entirely in response to major changes to the environment b) illustration of rate-remapping: place fields remain in the same location between sessions but change their peak infield firing rate in response to contextual cue changes

environment (Gothard *et al.* 1996, Shapiro *et al.* 1997, Knierim 2002). Both forms of remapping are thought to allow animals to disambiguate different environments, thereby reducing the amount of interference between similar experiences. Global remapping might allow differentiating between similar events that occur in different locations, while rate-remapping might enable differentiating between events that occur in the same location but are different in some salient way.

Place cells are capable of anchoring their fields to both distal and proximal cues as well as to olfactory cues and self-motion, although in most situations they may be responding to a combination of these. Cue-rotation experiments were performed by Muller and Kubie (1987) who found that if a cue-card was rotated around the environment, place fields would rotate to the correct location relative to the cue. In addition, place fields can be tied to proximal cues; Knierim and Rao (2003) found that place cells with fields on a circular track maintained their firing location relative to the track when it was moved around within the room, but would rotate around the track when the distal cues were rotated coherently around the track. Knierim (2002) also found that when distal and proximal cues were put in conflict with each other, some place cells would follow each set of cues, while others would remap or become silent. This suggested that the activity of an individual place cell may be more dependent upon the specific sensory inputs rather than all cells responding coherently as a hippocampus-wide network. The cue-rotation approach to understanding what kinds of sensory information can drive place field expression has also been used for olfactory cues. Zhang and Manahan-Vaughan (2015) created a 'constellation' of odours within an environment. In the absence of visual input, place fields rotated consistently with

the olfactory cues. Two facts suggest that place cells also receive self-motion information. Place cells can maintain their place fields in darkness, which suggests that path integration based on idiothetic cues such as vestibular inputs or proprioceptive information may be enough to support place field stability, although there was evidence that fields could drift if located far from a border (Quirk *et al.* 1990, Markus *et al.* 1994, Zhang *et al.* 2014). Also when cue rotations are performed when the animal is within the environment, place fields do not rotate to follow the cues unless the rotation is by a very small amount (45°) (Rotenberg and Muller 1997). This also suggests that visual information and self-motion information are both inputs and place fields will not follow the visual information if it conflicts with the internally generated sense of direction. Collectively these data suggest that place fields can be tied to all sensory inputs and may be supported by all or any of the remaining cues when deprived of one cue.

Place cells can encode more than just current location or 'place'. In O'Keefe and Dostrovsky's initial experiment (1971), they observed that a subset of place cells responded to place plus facing direction, or place plus a specific behaviour such as eating or exploring. Place cells have since been shown to encode many other aspects of the environment, particularly when these are salient.

In a linear track, place cells have been shown to fire in a direction specific way (McNaughton *et al.* 1983, Gothard *et al.* 1996). In the latter paper, rats were trained to shuttle from end to end of a linear track. 88% of place cells with fields on a linear track only fired when the animal was running in one direction. This directional modulation only occurs when animals' paths through a place field are constricted or limited to stereotyped paths through the place field. Taking this further, on tasks in which animals run through one location in the same direction, but as part of two or more different trajectories, a similar differentiation in firing rate between the different trajectories occurs (Wood *et al.* 2000, Frank *et al.* 2000). It is this 'trajectory-dependent activity' which will be investigated further in this thesis.

In addition, it appears that place cells only encode these extra features of a place when these features are relevant. Markus *et al.* (1995) analysed place cells for directionality during either random foraging or when navigating between a trained set of reward locations. They found that place cells became directional during the second task even though they were not directional during random foraging. The same has been observed of trajectory-dependent activity, which only develops when a task is learned but is not present during random foraging on the same maze (Stevenson 2011).

Place cells have also been found to encode entirely non-spatial things. For example, Wood *et al.* (1999) found odour-specific cells. Rats were trained to compare the odour of a scented sand-well with the odour of the previous sand-well, and only dig if the odours were different. In this task more than half of the pyramidal cells recorded responded to non-spatial aspects of the task such a specific odour, the fact that odours matched, or the action of approaching a sand-well irrespective of its actual location, and other cells responded either to place alone or to combinations of location and non-spatial variables.

Recently pyramidal neurons in the hippocampus have also been shown to encode time (MacDonald *et al.* 2011, Kraus *et al.* 2013). Rats sequentially performed an object exploration, then a delay period of a set length, followed by an odour sample period in which they had to choose whether to dig based on whether the odour matched the previously sampled object. A population of CA1 pyramidal neurons fired in sequence during the delay period with each cell representing a different latency since the start of the delay. Similar to the behaviour of place cells these 'time cells' could also disambiguate the different trial types during the delay (MacDonald *et al.* 2011).

The data described above, suggest that place cells do not form a merely spatial cognitive map but in fact represent current location along with any other spatial or non-spatial salient information relevant to the task that the animal is learning.

1.1.3 Hippocampal anatomy

The hippocampus is subdivided into the dentate gyrus (and hilus), CA1, CA2 and CA3. The hippocampus and entorhinal cortex are interconnected to form the trisynaptic

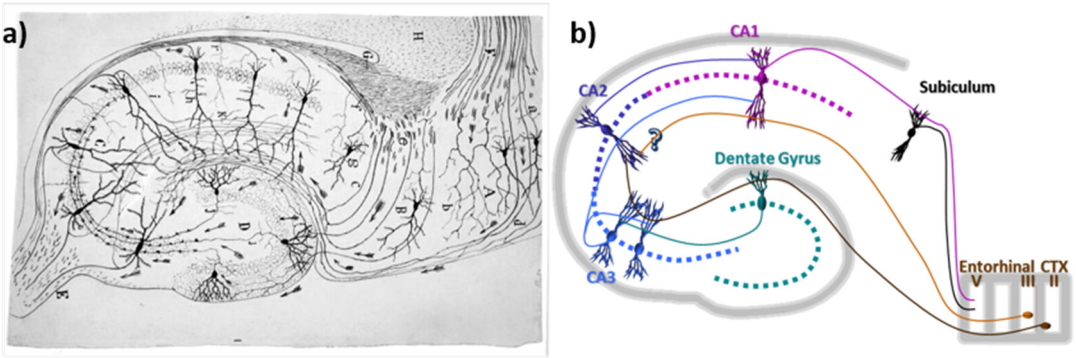


Figure 3: Hippocampus anatomy a) Cajal's original drawing of the trisynaptic circuit, b) a simplified diagram of the current knowledge of the connections within the hippocampus: LII EC (brown) projects to the Dentate Gyrus granule cells, and CA3 and CA2 pyramidal neurons. The granule cells (green) also project to CA3. CA3 (blue) projects to both CA2 and CA1 as well as forming recurrent connections to other CA3 neurons. CA2 (purple) projects to CA1. LIII EC (yellow) projects directly to CA1 (and possibly CA2). CA1 then projects to the subiculum and to the deep layers of EC.

circuit that was first observed by Ramon y Cajal using a Golgi stain to identify individual neurons and their projections (Figure 3a). The traditional trisynaptic circuit can be seen when the hippocampus is sectioned perpendicular to the septo-temporal axis. It begins with the Layer II entorhinal cortex projection to the granule cell layer of the dentate gyrus via the perforant path. These granule cells then project to the CA3 pyramidal cells via the mossy fibres. CA3 neurons then project via the Schaffer collaterals to CA1 pyramidal neurons. In addition there are many recurrent connections within CA3, which have been modelled as an attractor network which could allow associative memory (Rolls 1996, Levy 1996) or even generate place field-like firing patterns (Kali and Dayan 2000). In addition to the trisynaptic circuit, there is also a monosynaptic circuit; LIII of the entorhinal cortex projects directly to CA1 pyramidal neurons and it has been suggested that this projection is enough to support place cell activity in CA1 (Brun *et al.* 2002). Recently, the understanding of the connections within the circuit has been expanded to include area CA2 as well. CA2 receives inputs from both LII and LIII of the entorhinal cortex, as well as from CA3 and then projects on to CA1 (Jones and McHugh 2011). Figure 3b shows a simplified diagram of the current understanding of hippocampal connectivity. The hippocampus is also differentiated along the septo-temporal axis; inputs from the ventral entorhinal cortex project mainly to the ventral hippocampus, and inputs from the dorsal entorhinal cortex

project mainly to the dorsal hippocampus (Fyhn *et al.* 2004). In both the MEC and hippocampus there is a gradient of spatial precision, with cells in dorsal hippocampus showing smaller place fields than ventral hippocampus (Royer *et al.* 2010, Keinath *et al.* 2014), and dorsal MEC showing smaller grid spacing than ventral MEC (Fyhn *et al.* 2004). In addition, the LEC projection to CA1 targets distal CA1, while the MEC targets proximal CA1 (defined relative to the CA2-CA1 border) (Steward 1976).

1.1.4 Specialization of hippocampal subregions

The hippocampus is typically divided into the dentate gyrus, CA3, CA2 and CA1 areas. The different subregions have different proposed roles in navigation and memory due to their different cellular and connective properties. Subregion-specific lesions, and place cell activity patterns have also provided evidence backing up this idea and this literature will be summarized here.

The dentate gyrus: pattern separation

The main excitatory cell type within the dentate gyrus are the granule cells. These cells receive inputs from LII of the entorhinal cortex and project to both CA3 pyramidal cells and interneurons via the mossy fibres. The dentate gyrus has been proposed to play a role in spatial memory, particularly in spatial or episodic pattern separation. Pattern separation is the ability to disambiguate similar places or experiences from each other even when they share some features. Interestingly, the granule cell layer is unique in being a site of adult neurogenesis (Altman and Das 1965). The availability of new neurons could allow the dentate gyrus to encode new memories allowing the disambiguation of similar episodes or contexts. Consistent with this, new granule cells were found to be more active than older granule cells (Nakashiba *et al.* 2012). The authors also found that in mice in which older granule cells were inhibited relative to newer granule cells, pattern separation was enhanced while rapid pattern completion was decreased. It has been suggested that granule cell neurogenesis could provide a constantly changing population of cells to form distinct new representations (Kempermann *et al.* 2004). This might enable differentiation of temporally separated episodes, since the population of newly-formed granule cells will change over time allowing them to be disambiguated later. Dentate gyrus lesions have been shown to

cause navigation deficits in the watermaze (Jeltsch *et al.* 2001, Sutherland *et al.* 1983), and spatial pattern separation tasks (Gilbert *et al.* 2001, Morris *et al.* 2012). Dentate gyrus lesion animals are also impaired at object-context recognition but not object recognition (Dees and Kesner 2013). In addition, lesions of the ventral DG impair pattern separation between similar odours (Weeden *et al.* 2014). In summary the dentate gyrus seems to have a role in pattern separation or the ability to disambiguate similar experiences.

CA3: rapid association and pattern completion

CA3 receives inputs from both the LII of the entorhinal cortex, and the dentate gyrus. Unlike the rest of the hippocampus the major input to each CA3 pyramidal cell is other CA3 pyramidal cells (Amaral *et al.* 1990). CA3 neurons can make around 11,000 connections with other CA3 neurons (Wittner *et al.* 2007). These recurrent connections have been suggested to allow both associative learning and create place field activity (Hasselmo *et al.* 1995, Rolls 1996, Levy 1996, Kali and Dayan 2000).

The recurrent connections in CA3 have been suggested to allow associative learning. Lee and Kesner (2002) showed that plasticity in CA3 is necessary for learning new information in a novel environment, and Gilbert and Kesner (2003) showed that CA3 was needed for learning object-place associations and odour place associations but not object odour associations, suggesting that CA3 is only involved when there is a spatial component to the task. There is evidence that the associative network in CA3 allows rapid learning. It is needed for one-trial learning in a new environment (Lee and Kesner 2002, 2003). This is also backed up by immediate early gene data which shows increased Arc expression in CA3 but not CA1 after a single lap of a new environment (Miyashita *et al.* 2009). Similar effects are caused by infusing AP5 into CA3 or using a mouse line in which NMDARs are knocked out in CA3 (Lee and Kesner 2002, Nakazawa *et al.* 2002), suggesting that plasticity in CA3 is necessary for rapid learning. CA3 also projects directly to the medial septum via the fimbria, and this pathway may explain why lesions of CA1, which is sometimes thought of as the only output of the hippocampus, do not always produce deficits in hippocampus-dependent spatial tasks (Lee and Kesner 2003, Lee *et al.* 2005).

The structure of the CA3 network also suggests that it could enable pattern completion or the ability to retrieve a complete memory of an experience based on re-exposure to part of the experience. This would allow a subset of the cues originally present in an environment to reactivate the whole map of the environment. This idea was investigated by Vazdarjanova and Guzowski (2004) who used a combination of two immediate early genes to look at the amount of overlap between the populations of cells activated in two similar environments. They found that when there was little difference between the environments there was more overlap between the populations of CA3 cells active in the two environments than between the CA1 populations, suggesting that CA3 does not remap in the changed environment but encodes the two environments similarly, while CA1 cells show partial remapping. Electrophysiology also supports this, with the CA3 place cell maps remaining more coherent following inconsistent changes in cues than CA1 place cells (Lee *et al.* 2004). Similarly, Neunuebel *et al.* (2014) found that CA3 place cells showed a coherent response during a cue-conflict session, even though the dentate gyrus place cells showed a disrupted map. This backs up the idea that the associative network causes CA3 cells to remain coherent following a change in the environment by using pattern completion. This was tested behaviourally by Gold and Kesner (2005), who performed CA3 lesions with the result that animals performed worse than controls on a delayed match-to-place task, particularly when only a subset of the original landmarks was available. Like rapid learning, pattern completion also seems to require NMDAR-dependent plasticity in CA3. Mice without NMDARs in CA3, were able to perform normally in watermaze reference memory, but were impaired when some of the landmarks were removed (Nakazawa 2002).

CA3 neurons project to both ipsilateral and contralateral CA1 (Shinohara *et al.* 2012) but there is evidence that projections from right and left CA3 show different amounts of plasticity with projections from right CA3 showing little plasticity (Kohl *et al.* 2011). When the contributions of the left and right CA3 were compared behaviourally they found that silencing of right CA3 had no effect on an associative spatial long term

memory task (place learning on the Y-maze) compared with a deficit following left CA3 silencing (Shipton *et al.* 2014).

CA2: pattern separation or social memory?

Anatomically CA2 resembles a cross between CA3 and CA1. Similar to CA3, it receives input from LII entorhinal cortex, and its cells resemble the pyramidal neurons found in CA3. However, unlike CA3 and like CA1 it receives no input from the dentate gyrus, and receives inputs from LIII entorhinal cortex (Jones and McHugh 2011) although this has been disputed (Kohara *et al.* 2014). In addition it receives inputs from CA3 pyramidal neurons. CA2 projects to CA1 and has also been shown recently to project to MEC LII (Rowland *et al.* 2013). CA2 is thought to respond to differences between past and current experiences allowing pattern separation. An experiment using two different immediate early genes to look at the overlap between population of cells activated in two different epochs revealed that CA2 neurons showed significantly less overlap (suggesting global remapping) when a small change was made between the two epochs compared with the overlap seen in either CA1 or CA3 (Wintzer at al 2014). Recently it has also been hypothesized that CA2 is necessary for social processing (Hitti and Siegelbaum, 2014). Using a transgenic line to silence CA2 neurons, they found impairments in social memory. Specifically, without CA2, mice showed no memory for littermates compared with a novel mouse despite being unimpaired at social odour discrimination. This suggests a more specific deficit in social memory but does not rule out the possibility that CA2 has a role in spatial memory as well.

CA1: comparison, consolidation

CA1 is the major output of the hippocampus. It receives inputs directly from LIII entorhinal cortex, as well as indirectly via CA3 and CA2 inputs. Unlike CA3 it has no recurrent connectivity. It has been suggested that its connectivity with both the MEC and CA3 allow it to compare inputs from the entorhinal cortex, representing the current environment, with inputs from CA3, which might represent previously experienced environments.

Compared to CA3, CA1 cells show more remapping between different environments suggesting that pattern separation may lead to the CA1 place cell representation differentiating between environments. CA1 lesions have also been shown to cause a deficit in temporal pattern separation but not spatial pattern separation (Gilbert *et al.* 2001). Spatial learning in CA1 has also been shown to require NMDARs. Mice with knocked out NMDARs specific to CA1 neurons were impaired on the watermaze reference memory task (Tsien *et al.* 1996). Cabral *et al.* (2014a) also found that place cells showed increased place field size, however this was due to an increase in field 'jitter' between trials rather than a reduction in field precision within a trial. They suggested that this jitter was caused by an inability to store the place field location. Normally on each crossing of the place field, CA1 would compare ongoing inputs from MEC with inputs from the stored representation in CA3 and collectively these would create a stable representation. Without plasticity this process is impaired and a stable place field can only be formed after extensive exposure to the environment (Cabral *et al.* 2014a). This comparison between CA3 and MEC has been directly studied using a combination of recording and behaviour. LFP recordings can be used to infer communication between different brain areas. In CA1, high frequency gamma oscillations have been suggested to mediate MEC-CA1 interaction, while low frequency gamma oscillations mediate CA3-CA1 communication (Colgin *et al.* 2009). Cabral *et al.* (2014b) trained mice on a task which could be solved either through place learning or through learning a sequence of three egocentric turns. They found that normal animals could use either strategy and this was reflected in the place cell maps, which either encoded the turn sequence or the allocentric place. It appeared that the CA1 representation flipped between the two representations. Again, animals with NMDAR KO in CA1 neurons, showed an inability to form a map of the sequence of turns, but could form an allocentric map of the maze. The authors suggest that this reflects a reduction in the influence of CA3 inputs on CA1, because the KO mice also showed reduced low frequency gamma, and interestingly, did not show a shift in gamma frequency depending on the type of strategy used. This suggests that the deficits are caused by changes to the CA3 inputs rather than the MEC inputs to CA1 and suggests that the CA3 inputs are necessary for sequence learning.

CA1 has also been linked to intermediate term memory. Using a delayed non-matching to place task in a radial maze, Lee and Kesner (2002, 2003) showed that CA3 lesioned animals were impaired at acquisition with a 10 second delay between trials but CA1 lesioned rats were impaired at a 5 minute delay. An implication of this is that CA3 is needed for acquisition, perhaps because the environmental cues must be learned, but that after acquisition, CA1 can support task performance, perhaps through the direct inputs from the entorhinal cortex. In the watermaze, CA1 lesions performed 24 hours after training impaired memory but did not impair memory if performed 3 weeks after memory. This suggests that CA1 is necessary for a period after learning, after which the memory is stored elsewhere through systems consolidation (Remondes and Schuman 2004, Frankland and Bontempi 2005).

In summary then, the dentate gyrus receives spatial input from the MEC, and supports spatial pattern separation by forming a unique representation for each distinct episode, and passes information on to CA3, where associative learning occurs, allowing spatial and non-spatial aspects of episodes to be combined, as well as allowing different places to be associated together to form routes. CA1 is then in a position to compare inputs from CA3, which may represent memories of previously learned places, with inputs from the MEC, which may represent the current spatial input.

1.1.5 Trajectory-dependent activity

As was mentioned previously, place cells can represent the same place differently, depending on what trajectory an animal is taking through that place. Even in situations where the animal is travelling in the same direction through the place field, but is either coming from different start locations or going to different end locations, place fields can show rate modulation or field shifts between the different conditions.

This phenomenon was first observed in 2000 by both Frank *et al.* and Wood *et al.* on two different tasks. Frank *et al.* (2000) placed rats on a W shaped maze while recording from place cells in CA1. Rats were trained to run from the centre to one end of the W, back to the centre and then out to the opposite end of the W. Rats ran continuously and were rewarded for a correct choice at each end and the centre. A proportion of place

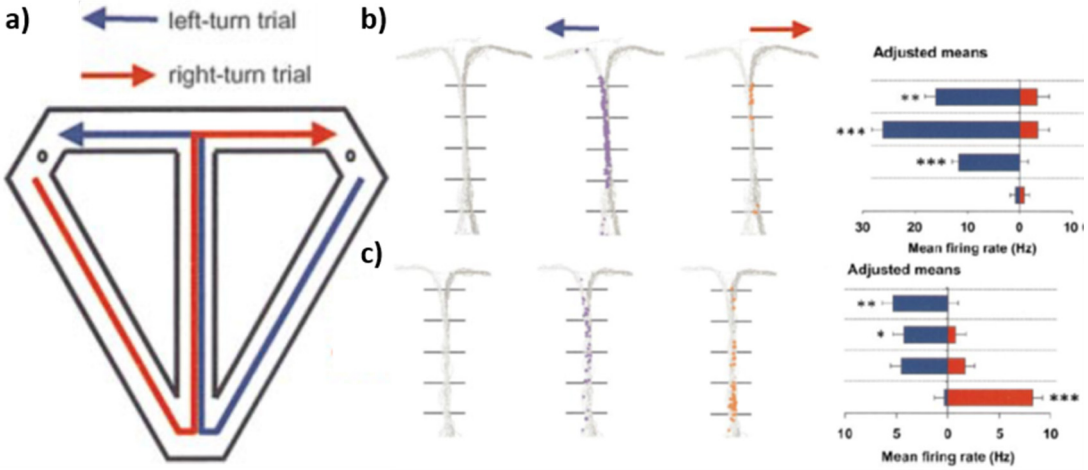


Figure 4: Trajectory dependent activity; a) the T-maze b) example of trajectory rate-modulation c) example of trajectory-dependent place field shifts

cells with fields in the central stem of the maze showed activity which differentiated between whether the animal had come from the left end or the right end on inbound trajectories, or which differentiated between whether the animal was going to the left or the right on outbound trajectories. They hypothesized that this activity might allow an animal to learn and plan extended trajectories through environments. Wood *et al.* (2000) recorded from CA1 place cells while rats ran a continuous alternation task on a T-shaped maze with returning arms (Figure 4). Animals would run up the central stem of the maze, turn right or left at the T-junction and then return to the start. They were rewarded for alternating each trial. In this task, they found that two thirds of place cells showed activity which differentiated between whether the animal was going to turn left or right (or had come from the right or left) either by rate-modulation (Figure 4b) or by shifting the place field location (Figure 4c).

This activity has been further characterized by Ferbinteanu and Shapiro (2003) and Bahar and Shapiro (2012) on the plus maze, by Holscher *et al.* (2004), Bower *et al.* (2005), Dayawansa *et al.* (2006), Smith and Mizumori (2006), Lee *et al.* (2006), Ainge *et al.* (2007b), Lipton *et al.* (2007), Ji and Wilson (2008), Pastalkova *et al.* (2008), Catanese *et al.* (2014) and Ito *et al.* (2015) on variants of the T-maze alternation task, and by Ainge *et al.* (2007a), Stevenson (2011), Huang (2010) and Grieves (2015) on variants of a serial reversal task on a double-Y-maze.

It has been suggested that trajectory-dependent activity occurs because the hippocampal representation flips between two or more different maps of the same environment (McNaughton *et al.* 1996, Toretzky and Redish 1996). Trajectory-dependent activity can be both prospective, representing differences in future trajectory, or retrospective, representing differences in past trajectory (Frank *et al.* 2000, Ainge *et al.* 2007a). If trajectory dependent activity represented the hippocampal representation flipping between maps, it would be expected that the map would flip between encoding the future trajectory and encoding the past trajectory but not show both at once. In the continuous T-maze alternation task it is not possible to separate out prospective coding from retrospective coding, so Catanese *et al.* (2014) used a visual discrimination task on the same maze in order to have good representation of each of the four combinations of trajectory start and end. By doing this they were able to compare the prevalence of prospective to retrospective firing while animals traversed the common central stem. They found more retrospective coding than prospective coding, and also that retrospective coding occurred more often on cells with fields in the first half of the central stem whereas prospective coding occurred more in cells with fields in the last third of the stem. However the two maps overlapped, with both prospective and retrospective fields active at the same time suggesting that a remapping theory is less likely to explain the data. Instead they proposed a buffer hypothesis, where prospective or retrospective activity reflects an episodic buffer representing the recent past or near future. Other evidence supporting this idea is that in the double-Y-maze one cell can have one field which shows trajectory-dependent activity while another remains modulated only by location (Grieves 2015). However this is also seen in other forms of rate-remapping where following changes to the environment which caused rate-remapping, different place fields of a place cell can show firing rate changes in the opposite directions (Leutgeb *et al*. 2005c, Leutgeb and Leutgeb 2007a). It therefore seems unlikely that trajectory-dependent activity is created by the hippocampal representation flipping between maps, but the activity could be explained by a process similar to rate-remapping.

One question that has been recently investigated is whether trajectory-dependent activity represents the whole trajectory or whether it represents the destination or goal of the animal. Ito *et al.* (2015) varied the continuous alternation task slightly by moving the reward site to the beginning of the central stem. The actual reward location or goal was therefore the same for both trial-types. Trajectory-dependent activity was still seen indicating that trajectory-dependent activity can encode the trajectory rather than just the goal location itself. Similarly Grieves (2015) showed that when the double-Y-maze was modified so that two trajectories led to the same goal, most trajectory-dependent activity differentiated between the two different routes to the same goal, also suggesting that the place cells were encoding the route taken rather than the goal.

Paper	**Task/maze**	**%**
Frank *et al.* 2000	Alternation: W-maze	36%
Ferbinteanu & Shapiro 2003	Place learning: Plus maze	59%
Wood *et al.* 2000	Continuous alternation: T-maze (B)	67%
Holscher *et al.* 2004	Continuous alternation: T-maze (NB)	8%
Lee *et al.* 2006	Continuous alternation: T-maze (B)	70%
Lipton *et al.* 2007	Continuous alternation: T-maze (B)	33%
Ainge *et al.* 2007b	Continuous alternation: T-maze (NB)	44%
As above	Delayed alternation: T-maze	4%(stem) 32%(delay)
Ito *et al.* 2015	Continuous alternation: T-maze (NB)	55%
Hallock & Griffin 2013	Continuous alternation/Visual Discrimination: T-maze	2%
Bower *et al.* 2005	Alternation: set path in circular arena (B)	37%
Lenck-Santini *et al.* 2001	Alternation: Y-maze	0%
Ainge *et al.* 2007a	Serial reversal: Double-Y-maze	44%

Table 1: Proportion of CA1 place cells showing trajectory dependent activity on each task B or NB indicates whether barriers were used (B) or not (NB) during training

Trajectory-dependent activity is not seen in all place cells that are active in a given environment, however the proportion of cells which show trajectory-dependent activity appears to vary even when animals are performing an identical task (Table 1). For instance, in the T-maze continuous alternation task, the proportion of place cells that showed significant trajectory-dependent activity varied from 18% to 70% depending on the exact experiment. One possible explanation for this specific variation is the presence of barriers during training. Experiments in which barriers were used during training (Wood *et al.* 2000 and Lee *et al.* 2006) both resulted in higher

proportions of place cells showing significant trajectory-dependent activity, compared to experiment in which barriers were never used (Holscher *et al.* 2004, Lipton *et al.* 2007). Barriers might act as sensory cues differentiating the overlapping experiences. Once training has occurred the bias towards place cells representing the two trajectories differently may remain even if the barriers are no longer present. In support of this idea, Bower *et al.* (2006) trained animals in a complex place sequence task without barriers, a continuous alternation task on the same arena with barriers, and one without a reward at the choice point. They found trajectory-dependent activity occurred in the task in which barriers were used during training even after the barriers were removed. However, since then several other studies have seen evidence of high proportions of trajectory-dependent activity without the use of barriers during training (Ainge *et al.* 2007b, Ito *et al.*2015). It is possible therefore, that other differences in training procedure or the environment may be responsible for the differences in trajectory-dependent activity. They also found trajectory-dependent activity in the task without a reward at the choice point. Several experiments have not seen evidence of trajectory dependent activity despite the task appearing similar to tasks in which trajectory dependent activity has been seen. Lenck-Santini *et al.* (2001) recorded CA1 place cells as rats performed a Y-maze alternation task but found no evidence of trajectory-dependent activity. Holscher *et al.* (2004) also saw very little evidence of trajectory-dependent activity in the T-maze, and Bower *et al.* (2005) did not observe trajectory dependent activity in a complex sequence task, which they trained without barriers. It therefore appears that trajectory dependent activity is not the automatic result of learning a spatial task in a maze, but depends upon the exact training protocol used.

1.1.6 What is the role of trajectory-dependent activity?

The presence of trajectory-dependent activity in spatial tasks leads to the question of whether trajectory-dependent activity is necessary to learn the task. If trajectory-dependent activity is necessary for task learning, it would suggest that the differential activity represents the memory for the task rules, enabling the animal to perform the task. Alternatively, trajectory-dependent activity may merely be a secondary effect of

learning the tasks which is not necessary for task performance but perhaps plays a role in episodic memory or additional learning within the environment.

An obvious problem with the idea that trajectory-dependent activity represents the memory for the task, is that it is clearly seen in tasks which are not hippocampus-dependent. The continuous T-maze alternation task used by Wood *et al.* (2000) does not require a hippocampus unless a delay is inserted before the common stem of the maze (Ainge *et al.* 2007b). This suggests that at the very least trajectory-dependent activity will occur even when trajectory encoding within the hippocampus is unnecessary for task performance. However it does not rule out the possibility that trajectory-dependent activity does represent the memory of the task, when that task is hippocampus-dependent, and that its continued presence merely indicates redundancy within the brain with multiple systems simultaneously processing the memory in different ways. Alternatively trajectory encoding may be processed elsewhere in the brain and be passed to the hippocampus so that it can be incorporated into the representation of space (Ito *et al.* 2015). This may be necessary in some tasks, but be present in other tasks to allow episodic memory, or flexible responses to changing experimental conditions.

To explore this question, Stevenson (2011) and Huang (2010) recorded place cell activity while rats either explored a maze randomly or learned a task whose acquisition is hippocampus-dependent (Stevenson 2011) although performance of the task if the task has been pre-trained is only mildly affected by hippocampus lesions (Ainge *et al.* 2007a). Stevenson and Huang therefore explored the development of trajectory-dependent activity as the task was learned and contrasted this with animals who learned a random foraging task on the same maze. They found that trajectory-dependent activity only occurred when a specific trajectory needed to be remembered, and also that it appeared at the time at which animals learned the task and began showing good performance. Similarly Smith et al. (2012) also found that 'context-specific' activity appeared on a plus maze place task as a task was learned but did not appear when no task was learned. Collectively, these data support the idea that trajectory-dependent activity might represent the memory for the task. Contrary to this

however, Bower *et al.* (2005) found that in a task which is presumably hippocampus-dependent (a complex allocentric place sequence task) trajectory-dependent activity did not necessarily occur unless the training strategy biased towards a trajectory dependent representation through the inclusion of barriers during training. Bahar and Shapiro (2012) found that the proportion of cells showing trajectory-dependent activity did not predict how well animals performed on a place memory task on a plus maze, as certain task variations induced more trajectory-dependent activity but with a reduction in task performance, although there was a reduction in trajectory dependent activity during individual error trials. This effect was also seen by Ferbinteanu and Shapiro (2003) on the plus maze and Ito *et al.* (2015) on the continuous T-maze. Collectively, these results suggest that the prevalence of trajectory coding in the hippocampus for a given task does not correlate directly to how well the task will be solved. However within a given task, the extent of trajectory coding on a given trial correlates to performance on that trial. This result fits in with the idea that maze tasks may be solved in different ways, some of which may not involve the hippocampus or trajectory dependent activity and therefore the amount of trajectory dependent activity will not correlate with task performance. However, during errors, hippocampal trajectory coding will be reduced. This may either be the cause of the drop in performance, or may simply reflect the inputs of other areas which are involved in the task.

The T-maze task does become hippocampus-dependent if a delay is introduced at the start of the central stem (Ainge *et al.* 2007b). It might be expected that if trajectory-dependent activity represents a way of disambiguating the two trajectories then it would be present during this delay. Consistent with this, Ainge *et al.* found trajectory coding in 32% of cells active during the delay. This has been confirmed by Pastalkova *et al.* (2008) during a delay in which animals ran on a running-wheel between alternations on a T-maze. Hallock and Griffin (2013) also observed evidence of trajectory coding during a delay despite not finding any evidence of trajectory-dependent activity as animals ran the central stem. Gill *et al.* (2011) also saw trajectory-dependent activity during a delay on the plus maze place task. Trajectory-

dependent activity therefore may be present in the hippocampus even when not needed, to allow an animal to cope with delays or distractions since when a delay is introduced, trajectory encoded occurs during the delay rather than during running the task. However, when Ito *et al.* (2015) introduced a delay into the task after animals had been trained without one he saw reduced trajectory-dependent activity during the delay but observed it as animals ran the central stem following the delay, suggesting that this aspect of trajectory-dependent activity is also affected by the previous experience on the maze.

No one has directly tested whether the emergence of trajectory dependent activity requires plasticity within the hippocampus. However it has been indirectly tested by Ito *et al.* (2015), who recorded from CA1 place cells while acutely inactivating the nucleus reuniens (an area which they suggest is the source of the CA1 trajectory-dependent activity in this task). Acute inactivation of the nucleus reuniens on a trial-by-trial basis reduced trajectory dependent activity without affecting place field activity. This suggests that trajectory dependent activity in CA1 is not learned but rather dependent upon ongoing inputs from the nucleus reuniens.

Another possibility, which has not been directly investigated, is that trajectory-dependent activity in different brain areas might represent different things. For example, trajectory dependent activity in the MEC may represent different inputs based on the trajectory. CA3 may represent different contexts, N.Re may encode forward planning of the goal, and CA1 may depend upon the inputs from all three areas or be involved in episodic memory storage for later.

In summary, trajectory-dependent activity usually occurs during tasks in which multiple trajectories pass through a common location. It also develops as a hippocampus-dependent task is learned. However the prevalence of this activity within the place cell population varies greatly depending on the specific task demands, or training protocols used. Therefore it seems likely that trajectory-dependent activity is a way for the hippocampus to either disambiguate overlapping episodes, or to maintain a trajectory in working memory but this representation is not always necessary for

correct performance. In tasks in which the trajectory can be solved by remembering a small number of simple body turns other brain areas (for example the striatum (Barnes *et al.* 2005) may be capable of supporting this memory. The fact that the proportion of trajectory-dependent activity seen varies with the task demands, and even with subtle differences in training or prior experience has implications for any study attempting to compare trajectory dependent activity in different conditions or brain areas, and suggests that comparisons should be made within animals or at least with as little difference in training protocol as possible between conditions.

1.1.7 Where does trajectory-dependent activity originate?

Another unanswered question about trajectory-dependent activity is where it originates. Trajectory-dependent activity has been observed in CA1, both deep and superficial layers of the MEC, and recently in CA3 (Frank *et al.* 2000, Lipton *et al.* 2007, Bahar and Shapiro 2012). Outside the medial temporal lobe, non-spatial trajectory encoding has also been observed in the mPFC (Ito *et al.* 2015), nucleus reuniens (Ito *et al.* 2015) and retrosplenial cortex (Smith *et al.* 2012).

The first paper describing trajectory-dependent activity found that a greater proportion of neurons in the MEC showed trajectory-dependent activity than in CA1 (Frank *et al.* 2000). Lipton *et al.* (2007) also found increased trajectory dependence in MEC neurons than CA1 neurons although they did not differentiate between deep and superficial layers. They also observed a reduction in spatial precision in MEC neurons compared with hippocampal neurons. Since the MEC, CA3 and CA1 form a circuit, it is not obvious where the activity might originate. Frank *et al.* (2000) observed a greater degree of trajectory-modulation from cells in the deep layers of the MEC than was observed in either superficial MEC or CA1. They suggested that this meant that weak trajectory-dependent activity in superficial MEC was combined with spatial information in place cells and finally was output to deep layers of the MEC.

The MEC projects both directly to CA1 and indirectly to CA1 via the dentate gyrus and CA3. Therefore it is possible that trajectory-dependent activity could originate in the MEC and be passed on to CA1 either with or without the presence of trajectory-dependent activity in CA3. Alternatively, trajectory-dependent activity might originate

in CA3, perhaps driven by inputs from the LEC, which are known to cause rate-remapping in CA3 in response to contextual changes (Lu *et al.* 2013). Or finally trajectory-dependent activity might originate in CA1, driven by inputs from other brain areas. CA1 receives inputs from the midline thalamic nucleus reuniens, which does not project to CA3 or the dentate gyrus, and trajectory encoding has been seen in nucleus reuniens neurons (Ito *et al.* 2015).

Two studies have currently looked for trajectory-dependent activity in CA3. Bahar and Shapiro (2012) observed that similar proportions of place cells in CA3 showed trajectory-dependent activity as in CA1. Contradicting this, Ito *et al.* (2015) observed much reduced trajectory-dependent activity in CA3 compared with CA1. Since both studies used similar methods of analysis and recorded from many individual place cells these results seem contradictory. One potential explanation for this difference is the tasks used in the two experiments. Bahar and Shapiro used a plus maze place-learning task. Animals were placed in varying start locations on the plus maze and had to rapidly learn to run to a constant reward location. This task is known to depend upon an intact hippocampus (Packard and McGaugh 1996). On the other hand, Ito *et al.* used a continuous alternation task on the T-maze. This task is known not to require an intact hippocampus (Ainge *et al.* 2007b). Collectively these studies suggest that trajectory-dependent activity is generated in different brain areas depending on the specific demands of the task. Since trajectory-dependent activity has not been recorded outside of CA1 in many different tasks, more research is necessary to confirm this hypothesis.

Since trajectory dependent activity develops (in CA1 place cells) during learning the serial-reversal task on the double-Y-maze and is not present during random foraging, trajectory dependent activity is potentially dependent upon a learning process. An ideal site for this learning is CA3 since it has been shown to be necessary for rapid associative learning and since CA3 place cells undergo rate-remapping in response contextual changes. This is particularly likely because hippocampal lesions do not greatly impair performance on the double-Y-maze task once it has been learned but do entirely block initial acquisition of the task. In addition the deficit that is seen in animals lesioned after training, is a deficit in switching between blocks. Both deficits

in learning the initial maze task (in a novel environment) and deficits rapid learning a new goal location (over the course of a few trials) are consistent with effects of CA3 lesions, making it likely that CA3 is involved in solving this task. Therefore this task may be a good place to test the hypothesis that trajectory dependent activity occurs in CA3 in hippocampus-dependent tasks despite not occurring in the continuous alternation task. This will be investigated in Chapter 2 of this thesis.

1.2 The role of the nucleus reuniens in navigation and as an input to CA1 place cells

The nucleus reuniens is a midline thalamic nucleus, which has recently gained interest as a possible relay between the medial prefrontal cortex (mPFC) and the hippocampus. Since nucleus reuniens neurons provide direct excitatory input onto hippocampal pyramidal neurons in CA1, it is also a possible source of the trajectory-dependent activity seen in place cells. This section will review the anatomical, behavioural, and electrophysiological evidence supporting the idea that the nucleus reuniens is a relay between the mPFC and the hippocampus, as well as summarizing the possible roles the reuniens plays in behaviour.

1.2.1 Anatomy of the nucleus reuniens

The nucleus reuniens is a small midline thalamic nucleus located just above the third ventricle (Figure 5). Together with the rhomboid nucleus, it makes up the ventral midline thalamus.

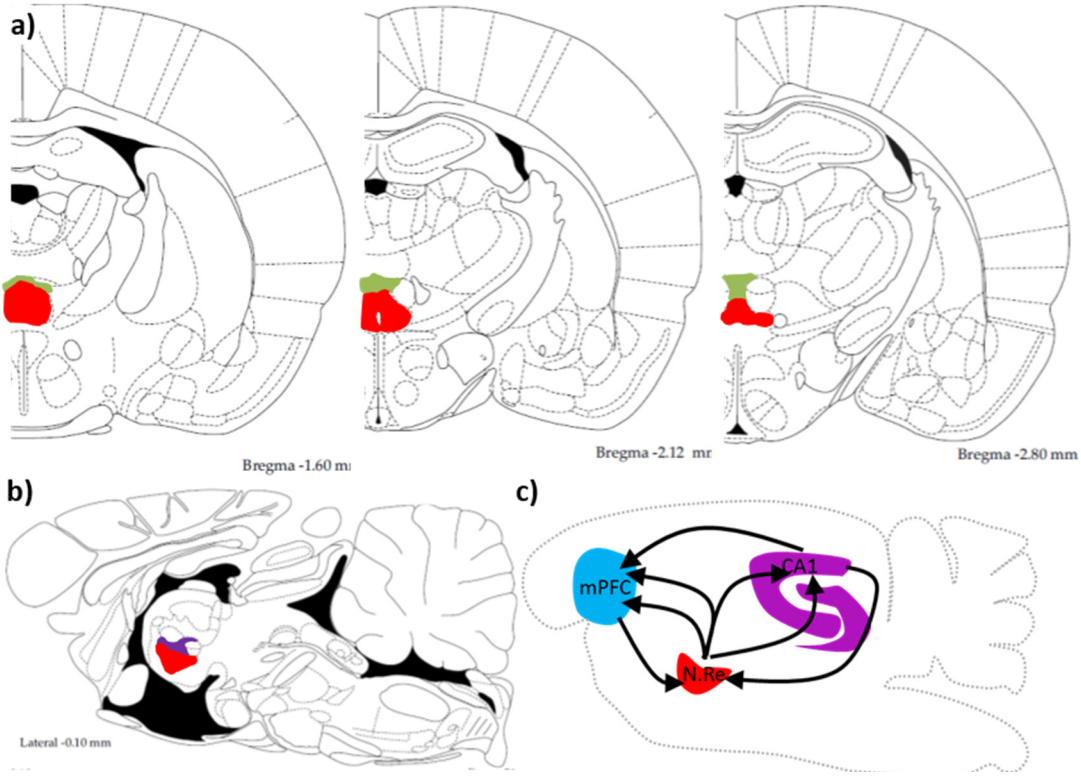


Figure 5: The nucleus reuniens a) location of the nucleus reuniens red and rhomboid nucleus green on coronal sections b) location of the nucleus reuniens in a sagittal section c) simplified connectivity of the nucleus reuniens with the mPFC and CA1

Efferent and afferent projections of the nucleus reuniens

It receives its main cortical inputs from the mPFC but also receives weaker projections from other cortical areas and diverse subcortical areas. The mPFC is thought to be involved in memory, (Farovik et al, 2008), decision making, strategy switching, and the representation of rewards or goals (Schroeder *et al.* 2001) and so this projection to reuniens might indicate that the reuniens also contributes to these processes. All of the four mPFC subregions; the medial agranular, anterior cingulate, infralimbic and prelimbic cortices, strongly project to the nucleus reuniens (Vertes 2002). The nucleus reuniens also receives projections from areas associated with spatial processing including CA1 and the subiculum but not the dentate gyrus or entorhinal cortex, and areas associated with processing orientation such as the lateral mammillary nucleus, postsubiculum and retrosplenial cortex (McKenna and Vertes 2004).

The nucleus reuniens projects back to the mPFC with dense projections throughout the infralimbic and prelimbic cortices, and weaker projections to the anterior cingulate cortex and medial agranular cortex (Vertes *et al.* 2006). It also projects strongly to CA1, but does not project to the dentate gyrus or CA3. In addition it projects to other areas involved in spatial processing such as the subiculum, presubiculum and the perirhinal and entorhinal cortices (Vertes *et al.* 2006, Dolleman-van der Weel and Witter 1996) and also projects to the nucleus accumbens (Otake and Nakamura 1998).

The reuniens as a relay between mPFC and hippocampus

Since the nucleus reuniens both receives projections and projects to the hippocampus and the mPFC, it is ideally situated to act as a relay between the structures. Unlike the strong mPFC to N.Re projections, there is no direct projection from the mPFC to the hippocampus. In addition only the ventral hippocampus projects back directly to the mPFC (Degenetais *et al.* 2003, Hoover and Vertes 2007). Double tracing studies in which different tracers are infused into both hippocampus and mPFC have revealed several interesting aspects of the nucleus reuniens connectivity. The projections from the reuniens to the hippocampus and mPFC are organized such that the anterior reuniens sends more projections to the hippocampus and the posterior reuniens sends more projections to the mPFC, while the middle of the nucleus reuniens strongly

projects to both areas (Hoover and Vertes 2012). It has also been shown that a proportion of reuniens neurons project to both the mPFC and the hippocampus (Hoover and Vertes 2012, Varela *et al.* 2014). Crucially, neurons from the mPFC form excitatory synapses directly onto reuniens neurons which project to CA1, confirming that the reuniens does constitute a disynaptic relay between the mPFC and CA1 (Vertes *et al.* 2007). Neurons from the nucleus reuniens have also been shown to form excitatory connections on both the pyramidal neurons and interneurons in CA1, suggesting that the reuniens can both excite or inhibit CA1 principle neurons (Dolleman-Van der Weel *et al.* 1997, Dolleman-Van der Weel and Witter 2000, Bokor *et al.* 2002).

The projection from the nucleus reuniens to CA1 has been tested with electrophysiology and results indicate that the reuniens projection is stronger than the projection from CA3 and shows greater plasticity in response to short-interval paired stimulations (Bertram and Zhang 1999). However this is inconsistent with Dolleman-van der Weel *et al.* (1997), who did not find evidence of CA1 pyramidal spikes in response to stimulation of the nucleus reuniens but only subthreshold depolarization.

The reuniens also has internal connections; with the posterior reuniens projecting to the anterior part of the nucleus, and thereby forming a disynaptic projection from posterior reuniens to CA1 (Dolleman-van der Weel *et al.* 1997). Stimulation of both anterior and posterior regions of the nucleus reuniens resulted in responses in CA1, but the timing of the response following posterior reuniens activation indicated a disynaptic connection whereas shorter response times for stimulation of the anterior reuniens indicated a monosynaptic connection, matching the anatomical data.

The anatomy of the nucleus reuniens strongly suggests that it may have a role in conveying information between the mPFC and CA1 in the hippocampus. In addition it may have a role in modulating the activity in both areas

1.2.2 The role of the nucleus reuniens in navigation and memory

Spatial learning

Since the reuniens projects strongly to the hippocampus, and potentially forms a relay between the mPFC and the hippocampus, many experiments have aimed to investigate the role of the reuniens in spatial tasks. The watermaze reference memory task is an established means of testing allocentric navigation. Animals are trained for multiple trials in which they are placed within a large pool of opaque water and must swim to find a hidden escape platform. This platform is kept in the same place across trials and can be located using the multiple cues surrounding the pool. As animals learn the task, their latencies to find the platform decrease. Probe trials in which the platform is removed allow measurement of how well the animals remember the platform location. Several different labs have tested the effects of nucleus reuniens lesions or inactivation on the watermaze reference memory task, with slightly inconsistent results. Firstly, Dolleman-van der Weel *et al.* (2009) found that nucleus reuniens lesioned rats were not impaired on a reference memory task in the watermaze. Their latencies to find a hidden platform in a constant location in the watermaze decreased both within and between days with latencies similar to those of control animals indicating that they were unimpaired at learning or navigating to the platform location. However the lesion group did show a difference in their response to probe trials in which the platform was not present. Reuniens lesion rats swam to the platform location initially, but upon finding that it was not there they began searching the pool randomly, in contrast to control rats who continued searching in the area around the platform location. This suggests that the reuniens is not needed to learn the reference memory task, but that it may be involved in strategy selection following a change to the task.

These results were confirmed by Loureiro *et al.* (2012) and Cholvin *et al.* (2013). Both papers report no deficit on initial place learning in the watermaze. Cholvin *et al.* also showed that although lesion rats spent significantly more time in the correct quadrant than the incorrect quadrants during probe trials, indicating memory for the platform location, their time spent in the correct quadrant was reduced compared to controls. This result could potentially be explained by the increase in random searching during

the probe trial seen by Dolleman-van der Weel *et al.* (2009). Contrary to these results, an inactivation study using tetracaine to block nucleus reuniens activity during either the learning phase, the consolidation phase, or during retrieval of the watermaze reference memory task found that rats were significantly impaired if the nucleus reuniens was inactivated at any of these stages of the task, although the results on the effects of inactivation during consolidation and retrieval are unreliable as control animals did not show significant memory for the platform location in the probe trial (Davoodi *et al.* 2009). The authors also found that nucleus reuniens inactivation impaired learning and performance of a delayed-match-to-place task in the watermaze. However since the authors did not attempt to control for or to measure the spread of the tetracaine it is possible that it spread beyond the nucleus reuniens and that the deficits reported in this paper are the result of inactivation to wider thalamic regions. Alternatively, the deficits seen may indicate that the reuniens has a role in place learning which is apparent following acute inactivation, but that other regions can compensate following a chronic lesion to the reuniens.

Memory persistence

Loureiro *et al.* (2012) extended the results of Dolleman-van der Weel *et al.* by looking at the effects of reuniens lesions on long term memory persistence. After training rats over 8 days to find a platform in a consistent location, they tested animals on their memory 5 or 25 days later. Although lesion rats were not impaired during initial learning nor at a delay of 5 days, they showed increased latencies to find the platform and reduced time spent in the correct quadrant during the 25 day delayed probe trial compared to control rats. This was followed up by another experiment in which the nucleus reuniens was inactivated only during the 25 day probe trial with no resultant effect on memory. Collectively these results suggests a role for the nucleus reuniens in the persistence of place memory over a long time but implies that it is not necessary for either the immediate encoding of the memory in the short term nor the retrieval of distant memories. The time-periods used in this study suggest that the reason for the deficit may be a failure in systems consolidation (Frankland and Bontempi 2005), and since systems consolidation is thought to require hippocampus to prefrontal cortex

communication it seems logical that reuniens lesions might impair this process by disrupting a major pathway of communication between these areas. Consistent with this, reference memory watermaze retrieval is dependent upon the mPFC after long delays (Teixeira *et al.* 2006, Lopez *et al.* 2012)).

Contextual fear conditioning is another task which can be used to study the transfer of memory between the hippocampus and mPFC. Xu and Sudhof (2013) investigated whether the nucleus reuniens had a role in memory specificity and generalization. Using fear conditioning, they showed that silencing the projections from mPFC to the nucleus reuniens caused overgeneralisation of contexts in fear memory. Rather than freezing only in the conditioned context, animals began freezing in a new contexts, suggesting that the contexts had been overgeneralized. This effect is also seen with prefrontal cortex silencing (Xu and Sudhof 2012), and non-generalised fear memory has also been shown to require the hippocampus (Wiltgen *et al.* 2010). Intriguingly, this effect was due to differences in acquisition rather than retrieval, as reuniens silencing after acquisition had no effect on subsequent generalization of the memory. The authors also found that optogenetically stimulating the reuniens at different frequencies could either cause reduced generalization or greater overgeneralization of the fear memory. Collectively, these results suggest the degree to which a fear memory is generalized is controlled by the mPFC-reuniens-hippocampus circuit. In summary, the reuniens appears to have a role in an aspect of memory that requires both the mPFC and the hippocampus and, similar to the watermaze results (Loureiro *et al.* 2012), suggest that the reuniens role in memory specificity occurs during acquisition rather than subsequent retrieval of memory.

The earlier results also suggest that the nucleus reuniens is involved in watermaze navigation but that it is not necessary for either learning or retrieving the platform location. Instead, the reuniens may be involved in strategy selection following changes to the task conditions and in increasing memory persistence over longer time periods.

Working memory

The role of the reuniens has also been investigated in non-watermaze-based tasks. These studies have generally been designed to investigate the role of the nucleus reuniens in either working memory or in strategy selection, with particular emphasis on tasks which require both the mPFC and the hippocampus. The results described above are consistent with the idea that the reuniens might not be needed for tasks which only require the hippocampus, since acquisition of the watermaze reference memory task, which is hippocampus-dependent but not mPFC-dependent (Sloan *et al.* 2006), is not affected by reuniens lesions.

Hembrook and Mair (2011) first tested the effect of nucleus reuniens on the Visuospatial reaction time task (VSRT), serial visuospatial reaction time task (S-VSRT), and a win-shift radial arm maze task (win-shift RAM), both continuous and with delays ranging between one minute and 30 minutes. They found that reuniens lesions caused deficits on the win-shift RAM but neither of the VSRT tasks. This result makes sense since neither the VSRT, which tests sensory guided learning, nor the S-VSRT, which tests action sequence learning, would be expected to require the hippocampus. The win-shift RAM task on the other hand, which requires animals to keep track of which arms they have entered, and so taxes working memory, is both hippocampus-dependent and mPFC-dependent (McDonald and White 1993, Porter and Mair, 1997, Mair *et al.* 1998). Consistent with this result Vann *et al.* (2000 a&b) found increased c-Fos expression in the nucleus reuniens and hippocampus in animals performing the win-shift RAM task compared with controls who merely ran up and down one arm. The authors did not measure c-Fos levels in the mPFC, however another study in mice did find increased c-Fos expression in both the mPFC and hippocampus following the working memory RAM task (Touzani *et al.* 2003). These results indicate that the mPFC, reuniens, and hippocampus all show increased neural activity during the RAM task. Hembrook *et al.* (2012) followed these results up by testing the effects of reuniens inactivation on two tasks which both require delayed conditional discriminations; namely the delayed nonmatching to position (DNMTP) in the operant chamber, and the varying choice delayed non matching (VC-DNM) in

the radial arm maze. Although these tasks seem similar, only the DNMTP is affected by both hippocampal and mPFC lesions, while the VC-DNM is only affected by hippocampal lesions (Porter *et al.* 2000, Mair *et al.* 1998). Consistent with their hypothesis, nucleus reunion inactivation impaired only the DNMTP task but not the VC-DNM task.

Strategy switching

As the mPFC is thought to have an important role in behavioural flexibility and strategy selection (Ragozzino *et al.* 1999, Ragozzino *et al.* 2003) it seems likely that tasks which require selection between strategies of navigation as well as having spatial memory component might require communication between the mPFC and hippocampus. Cholvin *et al.* (2013) used a newly designed task to test behavioural flexibility specifically applied to flexibility in navigation to a constant place when the start location changes. The double-H maze was first described by Pol-Bodetto *et al.* (2011) and is a watermaze in the shape of two adjoining H shapes (⧦). An escape platform is located in a constant location in the north-east arm during all trials. During training trials, animals are placed in either the north or the south middle arm and can reach the escape platform by turning either right then left, or left twice to reach the north-east arm. However during probe trials, animals are placed in the south-west arm and so must change strategy to reach the correct arm. Since the task was run in a maze with alleys, it was particularly easy to determine whether animals responded to a change in start location by navigating allocentrically to the original location, or whether they instead navigated egocentrically to an incorrect location by following the learned sequence of turns to arrive in the middle-top arm. They hypothesized that the task would require the mPFC and hippocampus and that it therefore might require the nucleus reuniens as well. During probe trials, control animals would usually follow the egocentric strategy initially to the north arm but would then search predominantly in the north-east (correct) arm by switching to an allocentric navigational strategy. In contrast, animals with either mPFC, hippocampus, or nucleus reuniens inactivation would swim to the incorrect north arm, but would then not shift to an allocentric strategy but would instead search randomly throughout the maze. Since the

hippocampus is needed for place memory, and the mPFC is needed for strategy switching, the most likely explanation for these results is that switching from an incorrect egocentric strategy to a correct allocentric strategy, requires the mPFC to perform the strategy change, the hippocampus to retrieve the correct place memory allowing the change to an allocentric strategy, and the nucleus reuniens to allow the necessary communication between these areas.

In summary, the most consistent difference between tasks that require the reuniens and tasks which do not, appears to be whether both mPFC and hippocampus are necessary. Both the radial arm maze task and the watermaze reference memory task are testing allocentric navigation and memory of locations. The difference is that in the watermaze task, one location is trained and tested over several days, and this location does not vary, whereas in the RAM tasks, the animal must keep track of multiple locations at once, and learn these locations during one trial. The non-delayed version is therefore testing working memory rather than reference memory, and it is this which makes the task dependent upon both the hippocampus and mPFC. The fact that reuniens lesions only affect tasks which are dependent upon both of the two brain areas to which the reuniens is strongly connected, but not tasks which only rely on one of them, implies that the reuniens has a role in allowing the two areas to communicate or modulate each other. If reuniens lesions merely caused a disruption to the function or activity of the areas to which it projects, then it would be expected that reuniens lesions would also impair hippocampus-dependent tasks such as watermaze reference memory. The results described above suggest that reuniens lesions do not affect hippocampus function, but only disrupt it in tasks which require interactions between the mPFC and hippocampus.

Attention or inhibition

The main conclusion of the majority of papers has been that the reuniens is involved in spatial working memory by providing a route of communication between the mPFC and the hippocampus. However, it has also been suggested that the reuniens is involved in modulating attention or inhibition.

Firstly, Chudasama and Prasad 2012 (see Mitchell *et al.* 2014 for review) tested the effects of nucleus reuniens lesions on the five choice serial reaction time (5CSRT) task. Reuniens lesions caused no change in performance at the standard version of the task, but when the task was made more difficult by reducing the length of the stimulus presentation, reuniens-lesioned rats surprisingly showed improved performance. The authors suggest that this result is due to reduced distraction, leading to an increased response accuracy, speed of response and reduced impulsive responses. These results potentially indicate that the method by which the reuniens produces the effects described earlier, such as long-term persistence of place memory in the watermaze or increased specificity of a fear memory is through increasing the detail of the hippocampal representation of the task or environmental cues. A possible mechanism for this is that reuniens excitation can increase the excitability of CA1 pyramidal neurons (Dolleman-van der Weel *et al.* 1997). This might allow weaker aspects of a memory to be encoded with more strength thereby adding detail to the representation (Xu *et al.* 2014). In some tasks this added detail is an asset but in other situations it may actually impair learning. Conversely, Prasad *et al.* (2013) suggested more of a role in inhibition, as reuniens lesions reduced the ability to inhibit premature responses but caused decreases in perseverative responses in the 5CSRT task.

1.2.3 What sort of information do nucleus reuniens neurons carry?

Recently Jankowski *et al.* (2014) found head-direction cells within the nucleus reuniens. In some ways this is not surprising since the reuniens receives inputs from three areas which contain head direction cells, the LMN, the retrosplenial cortex and the postsubiculum. However, the head-direction circuit has been described in detail as a predominantly linear pathway from the dorsal tegmental nucleus, to the LMN, anterior dorsal thalamus, postsubiculum and finally MEC. It is not clear where the nucleus reuniens fits into this pathway and what role if any the reuniens has on head-direction activity elsewhere in the head-direction circuit. Since head-direction coding was only observed in a very small proportion of recorded neurons (8.7%), there is no guarantee that the reuniens is providing head-direction information to the hippocampus but it is a possibility that should be explored further.

In addition, Jankowski also found a different population of neurons (4.3% of total recorded cells) within the nucleus reuniens which fired on alternate theta cycles. These showed a firing pattern similar to theta-skipping cells observed in the medial entorhinal cortex by Brandon *et al.* (2013). However unlike the cells found the MEC, the theta-skipping cells in the nucleus reuniens were not modulated by head direction.

In another interesting paper, Ito *et al.* (2015) describe trajectory encoding in nucleus reuniens neurons. The authors recorded from the mPFC, nucleus reuniens and hippocampus while animals ran a continuous alternation task in a figure-of-8 maze. A proportion of cells in all three areas showed trajectory modulation, in that they fired with a higher firing rate when the animal was running on one of the two trajectories through the central stem than the other. 38.2% of neurons in the mPFC (anterior cingulate cortex or dorsal prelimbic cortex), 43% of nucleus reuniens neurons and 55.1% of neurons in CA1 showed significant trajectory-dependent activity. The authors propose that the nucleus reuniens passes trajectory information from the prefrontal cortex to the hippocampus, where it can be combined with spatial information in place cells. This suggestion was backed up by the results of lesions or optogenetic inactivation of the nucleus reuniens. These manipulations both resulted in a large decrease in the amount of trajectory-dependent activity in CA1 place cells, suggesting that trajectory-dependent activity in CA1 is dependent upon ongoing inputs from nucleus reuniens neurons. They suggest that the mPFC via the reuniens provides the hippocampus with information about trajectory which might be necessary in tasks in which trajectory information must be combined with information about current location in order to make the correct choice of where to go. Interestingly Ito *et al.* (2015) did not observe head-direction activity in the recorded neurons, however given the relatively small proportion of significantly head-direction-modulated neurons observed by Jankowski *et al.* it is not particularly surprising that Ito *et al.* did not observe any head-direction cells in their recordings.

1.2.4 Summary and future work

In conclusion, the nucleus reuniens appears to have a role in mPFC-to-hippocampus communication. This connection has been shown through anatomy and

electrophysiology, but the functional relevance of the connection has also been tested, indicating that the link it provides is crucial for spatial working memory, memory consolidation, memory specificity, and strategy selection. The nucleus reuniens has also been shown to carry trajectory-dependent activity to the hippocampus in a spatial alternation task, but whether this input is important for learning or behaviour has not been investigated. Chapter 3 of this thesis will explore the role of the nucleus reuniens in a task which is known to induce the trajectory-dependent activity in the hippocampus. Additional aspects of the nucleus reuniens' role in several navigational tasks which require the hippocampus and/or the mPFC will also be investigated.

1.3 The role of the medial entorhinal cortex in navigation and place cell activity?

The major input to the hippocampus comes from the entorhinal cortex. As mentioned previously, the hippocampus and entorhinal cortex form a circuit with the entorhinal cortex acting as both the input and output of the hippocampus. The superficial layers project to the hippocampus, and the deeper layers receive inputs from the hippocampus. The entorhinal cortex is subdivided into the lateral and medial entorhinal cortices, which each contribute different information to the hippocampus. It is thought that the MEC provides more spatial information and the LEC provides more contextual information. This section will predominantly focus on the MEC, and its role as spatial input to hippocampal place cells, but will briefly touch upon differences between the LEC and MEC where it is relevant to their respective roles in spatial tasks. This section will first review the suggested role of the MEC in behaviour, then describe its anatomy, particularly the different projections to the hippocampus, and the morphological and functional cell types involved in this projection. Finally, it will describe different models of place field formation, and summarize the results of studies which have sought to test these models experimentally.

1.3.1 What is the role of the MEC in navigation and memory?

Since the MEC receives inputs from several areas involved in spatial processing particularly the postrhinal cortex and postsubiculum, while the LEC receives inputs from the perirhinal and piriform cortices, it has been suggested that the MEC provides the hippocampus with spatial information about an environment, while the LEC provides information about the content of an environment (Burwell and Amaral, 1998, Aggleton *et al.* 2000). This difference has been tested in several different ways.

Lesions to the MEC result in deficits in place learning in the watermaze task (Hales *et al.* 2014), but lesions to the LEC do not have this effect (Ferbinteanu *et al.* 1999, Van Cauter *et al.* 2013). The evidence that the MEC is involved in watermaze reference memory is somewhat contradictory. Several studies have found deficits on the watermaze reference memory task (Schenk and Morris 1985, Oswald and Good 2000, Van Cauter *et al.* 2013, Hales *et al.* 2014), however others have found no deficit

(Pouzet *et al.* 1999, Burwell 2004). One possible explanation for these differences is the specific regions of the MEC that were lesioned. Papers which found navigational deficits tended to involve lesions of the dorso-caudal MEC whereas papers with no deficits tended to show sparing of this region (Morrissey and Takehara-Nishiuchi 2014). In papers showing deficits, MEC lesioned animals do show learning of the platform location over multiple days of training as shown by decreased latencies to find the platform and significantly increased dwell time in the platform quadrant on probe trials. However, they show lasting impairments, with longer latencies and reduced time in the target quadrant compared to control animals even after multiple days of training (Van Cauter *et al*. 2013, Hales *et al.* 2014). Interestingly, in a paper which compared the use of distal and proximal cues, entorhinal cortex lesioned rats were impaired only on a version of the watermaze task in which the available cues were distal landmarks outside the maze, but were not impaired when proximal cues were placed in the water within the maze (Parron *et al.* 2004). Similarly a task in which the platform is located a set distance and direction from one proximal cue placed within the watermaze, rats with MEC lesions learned the task but showed no decrease in performance when the cues around the maze were removed, in contrast to control animals who showed a deficit when extramaze cues were removed (Oswald and Good 2000). Similarly in the standard watermaze task when the extramaze cues and room geometry were changed following 5 days of training on one platform location, lesion animals still showed significant dwell time in the quadrant which previously contained the platform, while the dwell time for sham animals dropped to chance, again suggesting that lesion animals were not using the distal cues to navigate (Hales *et al.* 2014). Collectively these results suggest that the MEC is involved in learning and performing the watermaze task based on the triangulation of extramaze cues, but that alternative strategies may allow navigation to the platform, perhaps by using proximal cues.

Similarly, using spontaneous object exploration tasks, it has been shown that MEC lesions do not affect object recognition, but do reduce the ability to remember the location or context of an object, whereas LEC lesioned animals show impaired

memory for object recognition (when there are more than 2 objects in total), object-place memory, and object-context memory (Save *et al.* 2004, Van Cauter *et al.* 2013, Wilson *et al.* 2013a&b, Hunsaker *et al.* 2013). An alternative method of testing the contributions of the medial and lateral entorhinal cortices to object and place memory was developed by Hunsaker et al 2007. They used either APV or naloxone, which block plasticity of the medial performant path and lateral performant path respectively, in different subregions of the hippocampus. In general they found that blocking plasticity of the MEC input to the hippocampus resulted in loss of object-place memory, while blocking plasticity of the LEC input blocked object memory. However it appeared that the MEC input to CA3 was necessary for object memory and the LEC input to CA3 and dentate gyrus was necessary for memory of the object's location. The results from spontaneous exploration studies collectively indicate that the MEC is necessary for detecting spatial changes, but the LEC is necessary for detecting non-spatial and in some cases spatial changes (Van Cauter *et al.* 2013). Hunsaker *et al.* 2007. The LEC and MEC are interconnected (Burwell 2000) so it is possible that for more complex object-location memory they interact, leading to deficits when either is damaged.

It has also been suggested that the MEC is involved in path integration. Path integration is a form of navigation that relies on keeping track of self-motion to identify your current position or to compute a homing vector without using external cues. It is usually tested using homing tasks, but the term also applies to the ability to keep track of the distance travelled, or to maintaining an internal sense of direction in the absence of external cues. It has been reliably shown that animals can use path integration to navigate (see Etienne and Jeffery 2004 for review). Van Cauter *et al.* (2013) tested this using a path-integration task on a Barnes-maze. Rats had to leave a 'home' box, and search for a piece of food in one of seventeen cups distributed around the maze. They then had to return to the home box. MEC lesions, but not LEC lesions, caused a reduction in correct returns to the home box, indicating that the animals had a path-integration deficit. Since MEC cells have been shown to respond to angle, speed, and distance, they might provide the perfect metric for this sort of computation

(McNaughton *et al.* 2006). If this were the case, MEC lesions would be expected to impair place cells more in the absence of visual information when the animal must rely more on path-integration to identify their location. However this has not been tested. A possible confounding factor is that rodents are particularly sensitive to smell and can maintain place fields based on odour cues alone (Zhang and Manahan-Vaughan 2015), even when these are only self-generated odours (Save *et al.* 2000), and so removing all non-self-motion cues is practically impossible.

Kneirim *et al.* (2014) have proposed that rather than the medial and lateral EC coding 'what' and 'where' respectively, both the MEC and LEC might both provide different types of spatial information. The MEC might act as part of a "global holistic spatial map" which provides information about current location and their relation to destinations, while the LEC encodes local item-location associations. This fits with electrophysiological recording data from both brain areas. Highly spatial or directional activity is found within the MEC (Fyhn *et al.* 2004) and typically forms a global representation of space (Neunuebel *et al*. 2013). LEC neurons on the other hand show less spatial modulation although they do respond to rotations of the local environment (Neunuebel *et al.* 2013) suggesting that LEC is not entirely non-spatial, but rather responds to local aspects of space. Similarly, Deshmukh and Knierim (2011) also found evidence that LEC neurons respond in a spatial way in environments containing objects. These were not simply 'object' cells since some cells represented locations which did not contain objects, while others fired in response to an object being taken away, suggesting that they might encode object-location. Collectively these results suggest that the MEC has a role in spatial navigation, spatial memory, and path integration, but that the LEC may have a complimentary role in spatial rather than being entirely non-spatial as was previously suggested. The different functional cell types found within the MEC and their possible roles in navigation will be discussed in more detail later.

1.3.2 **Anatomy: MEC as an input to the hippocampus**

Similar to the rest of the cortex, the MEC is divided into six layers. Layers I and IV do not contain many neurons. Layers II and III are the two superficial layers containing

neurons that are known to project to the hippocampus. Layers V and VI receive inputs from the CA1 and subiculum and are thought to be the main output of the MEC and hippocampus, as they contain projections to widespread cortical areas as well as the septum, striatum, amygdala and thalamus (Witter *et al.* 1989, Kerr *et al.* 2007). The MEC also receives inputs from diverse cortical areas such as the parietal, temporal and prefrontal cortices, and parahippocampal areas such as the subiculum and postsubiculum. It also receives inputs from the medial septum, several thalamic nuclei, the amygdala and the claustrum. These inputs predominantly target the superficial layers (Burwell and Witter 2000, Kerr *et al.* 2004). There is are also interconnection between the layers of the MEC, with projections from both superficial and deep layers to the superficial layers. Intrinsic connections are predominantly arranged in bands from dorsal to ventral MEC such that neurons in one band are not connected to neurons in the other bands, and the projections to the hippocampus also follow this pattern with dorsal MEC projecting to dorsal hippocampus (Dolorfo and Amaral 2007).

Layer II contains two excitatory cell types; stellate cells and pyramidal cells. It receives inputs from the postsubiculum and parasubiculum, LEC, CA2 and sparse projections from CA1 and the subiculum. It also receives inputs from within the MEC from layer III and layer V neurons (Rowland *et al.* 2013). The distribution of the two excitatory cell types within LII is not uniform; pyramidal cells are clustered into 'islands' surrounded by the stellate cells also called 'ocean cells' (Kitamura *et al.* 2014). Pyramidal cells project to both ipsilateral and contralateral CA1 where they innervate GABAergic interneurons. This projection appears to indirectly inhibit CA1 pyramidal neurons (Kitamura *et al.* 2014). The stellate cells project via the performant path to the dentate gyrus and CA3 where they form excitatory connections with granule cells and pyramidal cells respectively. This connection differs between rats and mice. In rats projections from the MEC to dentate gyrus are bilateral, while in mice they are ipsilateral only (van Groen *et al.* 2002). Also there is some evidence that LII does not project to CA3 in mice but only to the dentate gyrus (van Groen *et al.* 2003) however this has been contradicted by Suh *et al.* (2011) who found no evidence that LIII

projects to CA3 in the mouse. Stellate cells also interact with each other via inhibitory interneurons (Couey *et al.* 2003).

Layer III contains pyramidal cells which project to the hippocampus via the temporoammonic path to CA1 and subiculum (Steward and Scoville 1976). In both rats and mice this projection is bilateral although there are more ipsilateral projections than contralateral projections (van Groen *et al.* 2002, 2003). As with the LII input, this input is also arranged so that dorsal (caudal) MEC projects to dorsal hippocampus, and ventral MEC projects to ventral hippocampus. In addition, projections from the MEC project to the proximal half of CA1, while those from the LEC project to the distal half of CA3.

Layers V and VI are the deep layers of the MEC. The majority of projections from the hippocampus to the MEC project to these layers. Most projections originate in CA1 and the subiculum. There are predominantly excitatory projections from the deep layers to the superficial layers (van Haeften *et al.* 2003).

In summary, the overall connectivity of the entorhinal cortex is highly organized, both in the different projections into and from the MEC and LEC, and differences along the dorso-ventral axis of the MEC. This organization is also maintained in the pattern of projections into the hippocampus. Most relevant to the spatial role of the MEC, the more spatial inputs preferentially target the dorso-caudal MEC which is also the location of precise grid cells. This region of the MEC then projects to the dorsal hippocampus whose neurons show the most precise place fields. This anatomical organization also matches behaviour, since lesions of the spatially-precise region of MEC cause more profound navigational deficits than lesions of other parts of the MEC (Morrissey and Takehara-Nishiuchi 2014).

1.3.3 What information could MEC contribute to the hippocampus?

Grid cells

In 2004, Fyhn *et al.* discovered that a proportion of cells in the MEC fired in a very precise spatial way. Unlike place cells which typically have one or a few fields in a given environment, ‘grid cells’ form multiple repeating firing fields arranged in

repeating a triangular grid. Recordings throughout the dorso-ventral axis of the MEC revealed that grid cells in the dorsal MEC showed close spacing of the grids, while in ventral MEC grid cells the spacing was much further apart to the extent that grids could not be observed in small environments. This gradient of spatial precision matches the inputs, with the main spatial inputs from postsubiculum and postrhinal cortex projecting to dorsal MEC with less spatial inputs from perirhinal cortex projecting to ventral MEC (Burwell 2000). It was also observed that this gradient is not continuous but rather that grid cells are organized in modules, each with a certain grid spacing (Stensola *et al.* 2012). Grid cells within a module respond coherently to changes in the environment, but different modules may respond differently to changes in the environmental geometry. Grid cells are not dependent upon visual input but maintain the grid of firing fields even in darkness (Hafting *et al.* 2005), presumably indicating that grid cell activity can be maintained by self-motion cues. However they are influenced by visual cues, as the grid will rotate following a visual cue (Hafting *et al.* 2005). Grid cells have also been recorded in the presubiculum and parasubiculum (Boccara *et al.* 2010), and have also been recorded in bats and humans within the MEC (Doeller *et al.* 2010, Yartsev *et al.* 2015).

Grid cell activity has been modelled as a precursor to place cell activity, but there is some evidence that grid fields may actually depend on place cell inputs: Bonnevie *et al.* (2013) showed that following hippocampal inactivation, grid cells lose their grid lattice firing fields and their firing becomes head-direction modulated. Grid cell activity patterns also seem to be dependent upon the theta oscillation, since inactivation of the medial septum which provides theta-modulated input to the MEC, causes both a loss of theta modulation and grid field firing patterns in the MEC (Brandon *et al.* 2011, Koenig *et al.* 2011). However grid cells have been recorded in bats, who have no theta rhythm, suggesting that theta may not always be necessary for grid-like firing patterns (Yartsev *et al*. 2015).

It has been suggested that the repeating nature of grid cell firing patterns allows path integration. However, grid cells do not always show a constant spacing as would be the case if they were measuring distance. When recorded in boxes of decreasing size,

the field spacing decreased (Barry et al. 2007). In environments of the same size, grid fields become more widely spaced in a novel environment with different contextual cues (Barry et al. 2012). These results suggest that the MEC grid cells do not code an absolute distance measurement between locations but rather scale their firing patterns in response to novelty.

Head-direction cells

Head direction cells are found throughout the MEC. They are not modulated by an animal's location but fire with a high firing rate when the animal's head is facing in one direction irrespective of location. They were first recorded in the postsubiculum (Taube *et al.* 1990), but have also been recorded in the lateral mammillary nucleus, anterior thalamic nucleus, lateral dorsal nucleus, nucleus reuniens and retrosplenial cortex. The postsubiculum (also called the dorsal presubiculum), projects strongly to the superficial layers of MEC and presumably contributes the information necessary for MEC head direction cells (Taube 2007). Head direction cells typically respond coherently to changes in cues, with all head direction cells rotating their preferred firing direction by the same amount (Taube and Burton 1995, Yoganarasimha *et al.* 2006, Hargreaves *et al.* 2007). The regions containing head-direction cells form a pathway from the LMN to the ATN, then to the postsubiculum which then projects to the MEC. Lesions of any of these regions, remove head-direction firing from downstream regions but not upstream regions (Goodridge and Taube 1997, Blair *et al.* 1999). In early regions, the head-direction signal is controlled by self-motion alone, but from the ATN onwards, head-direction cells are anchored to visible cues in the environment and will rotate their preferred firing angle following rotations of the cues. Interestingly, lesions of the postsubiculum, prevent landmark control of head-direction cells in the ATN despite not removing head-direction activity itself (Goodridge and Taube 1997). Conversely, lesions of the MEC do not disrupt the ability of ATN head-direction cells to anchor to landmarks (Clark and Taube 2011).

The head-direction pathway ends in the MEC (Taube 2007), however it is likely that this directional signal is then passed on to the hippocampus, contributing crucial directional information to place cells. In support of this idea, lesions of the

postsubiculum prevent the anchoring of place cells to landmarks (Calton *et al.* 2003) suggesting that place cells require information either directly from head-direction cells in the MEC or from grid cells or conjunctive cells which may themselves depend upon the head-direction input from the postsubiculum.

Conjunctive cells

Conjunctive cells are a combination of head-direction cells and grids. They fire in a grid pattern like grid cells, but their firing is also modulated by the animal's head-direction (Sargolini *et al.* 2006). These are less common than either grid cells or head-direction cells.

Border cells/boundary vector cells

Border cells or boundary vector cells (BVCs) are found throughout the MEC (Solstad *et al.* 2008) as well as in the subiculum (Barry *et al.* 2006, Lever *et al.* 2009) and presubiculum (Boccara *et al.* 2010). They show high firing along any boundary that faces a certain direction. They can also fire parallel at a set distance from boundaries rather than immediately adjacent to such boundaries. Like head-direction cells, boundary cells rotate coherently following cues (Solstad *et al.* 2008).

Other cell types

Recordings within the MEC have also revealed several other functional subtypes. 'Speed cells' which respond to the running speed of the animal have been recorded in the dorsal MEC (Kropff *et al.* 2015). There have also been several reports of spatial non-grid cells within the MEC (Quirk *et al.* 1992, Brandon *et al.* 2011, Koenig *et al.* 2011). It is possible in some cases that the recorded cells could be grid cells but with such a large scaled lattice that only one field is seen in an environment. However it is likely that in addition to the grid cell population, there is also a population of cells which are spatially modulated but do not show repeating grid fields.

How does the anatomy match the function?

Since there are multiple morphological cell types within the superficial layers of the MEC and multiple functional cell types, it is an attractive idea that the functional cell types each correspond to particular morphological cell types. It has been suggested

that grid cells correspond to pyramidal cells in LII MEC, with border cells corresponding to stellate cells (Brecht *et al*. 2012, Tang *et al.* 2014). However recent evidence suggests that this is not the case and that all the functional cell types are found within each morphological population (Sun *et al*. 2015).

Zhang *et al.* (2013) used retrograde tracers and optogenetics to identify hippocampal projecting neurons within the MEC and confirmed that all the functional cell types do in fact project to the hippocampus. 27% of the projections they recorded were found to be grid cells, 12% were border cells, 7% were head direction cells 2% were spatial cells and 51% appeared to show no spatial modulation. This suggests that the MEC provides the hippocampus with multimodal spatial information, including information about boundaries, orientation and location.

1.3.4 Are grid cells or BVCs necessary for place field formation?

Grid cells have been proposed to form the precursor of place cells. It was proposed that the summation of grid fields together could lead to unique place fields. Several different models have confirmed that summation of grids of different sizes (and orientations) can summate to form place field-like activity (Fuhs and Touretzky 2006, Rolls *et al.* 2006, Solstad *et al.* 2006, Savelli and Knierim 2010, Lyttle *et al.* 2013).

In support of these models, it has been shown that remapping of grid fields leads to hippocampal remapping (Monaco and Abbott 2011). Also most spatially precise place cells are found in the region of the hippocampus which receives the most precise grid cell input. However if grid cell firing patterns were necessary for place field formation, then it would be expected that grid field activity would develop at the same time or before place field activity. Contradicting this, Langston *et al.* (2010) found that grid field activity in fact developed a few days after place cell activity. This result was also observed by Wills *et al.* 2010). As mentioned previously, medial septum inactivation abolishes grid cell firing patterns however this does not abolish place cell firing (Brandon *et al.* 2011 Brandon *et al.* 2014). Removing the grid-pattern input has no effect on the formation of a new hippocampal place map in a novel environment, nor on its duration across days. Also grid cells expand their firing grid size in response to

a novel environment (Barry *et al.* 2012). If grid cells were the main input driving place field formation, it would be expected that place field would also shift during this period, however place fields actually become stable quicker than the grid fields, suggesting that they are not controlled by the expanded grid field input. These results suggest that the grid to place cell models are not compatible with the experimental data.

One of the first proposed models of place field formation was the boundary vector cell model proposed by O'Keefe and Burgess in 1996, before the discovery of border cells/boundary vector cells in the subiculum or MEC. Their model proposed that place field formation occurs by the summation of several inputs each coding a set distance from boundaries facing in a specific direction. The model was tested by recording place cells in various rectangles of different aspect ratios. Place fields were found to respond to manipulations of the dimensions of an environment in the way predicted by the model with fields which lengthened as the environment was stretched in one direction. Later, Lever *et al* (2009) found boundary vector cells in the subiculum. The subiculum is now known to project to CA1 making this a potential input capable of supporting place field formation (Sun *et al.* 2014). Border cells were also identified in the MEC (Solstad *et al.* 2008) providing another possible pathway through which border cells could drive place cell activity. Since the boundary vector cell to place cell model could be supported by either the MEC inputs or the subiculum inputs this model does not require an intact MEC for the formation of place fields in the hippocampus (or at least CA1 place fields since it is not clear that the subiculum projects to CA3).

1.3.5 Is the MEC necessary for place field formation?

Both of the models of place field generation described above suggest that the MEC inputs (and/or the subiculum inputs) support place field formation. However, this has been contradicted by experimental research. Miller and Best (1980) were the first to record place cells from animals with entorhinal cortex lesions. They recorded place cells in rats as they navigated a radial arm maze and found that place cells still showed place fields, as each cell would fire in one arm of the maze and not in the others. However when the maze was rotated relative to the room, place fields in control

animals remained in the same place relative to the room, while place fields in lesion animals rotated with the maze. This provides an indication that MEC lesions may disconnect place cell activity from the distal cues.

This was followed up by 3 further lesion studies targeting all or some of the MEC (Brun *et al.* 2007, Van Cauter *et al.* 2008, Hales *et al.* 2014). Brun *et al.* (2007) selectively lesioned LIII of the MEC using γ-acetylenic GABA a neurotoxin which spares other layers of MEC. This lesion reduced the spatial information and stability of place cells. Van Cauter *et al.* (2008) lesioned all layers of the MEC and also found reductions in stability, but found that place field size actually decreased in the lesion group. In addition, they performed object rotation and removal sessions. All place fields in control animals rotated with the cues, in contrast to only 49% of place cells in the lesion group. However place fields remapped in the lesion group following cue removal in contrast to sham animals who were relatively stable. Hales *et al.* (2014) performed complete MEC lesions, specifically aiming to hit the most dorsal region of the MEC which contains precise grid cells. Their results match the previous results showing decreased spatial precision, decreased stability, and a decreased peak firing rate due to drift of the place field over time. In addition, they also showed reduced theta power, and a reduction in theta phase precession (Schlesiger *et al.* 2015). Since phase precession causes place cells to fire in close succession as animals run through their fields, it has been suggested to facilitate learning or retrieval of sequences of place cells through enhanced spike timing dependent plasticity (Byrnes *et al.* 2011).

One possible reason for the conflicting results on place field precision observed by these papers is the area of MEC that was spared. It has been suggested that the lesions in Van Cauter *et al.* (2008) spared the more dorsal region of the MEC, which provides the most closely spaced grid field input to place cells (Ormond and McNaughton 2015). According to one model of grid field to place field formation, ventral MEC lesions should cause a reduction in place field size, while dorsal MEC lesions should cause an increase in place field size. This was tested by Ormond and McNaughton (2015), who compared the effects of ventral and dorsal inactivation of the MEC. As expected, dorsal inactivation caused an increase in place field size, but surprisingly,

ventral lesions did not cause a decrease in field size, although they did show less increase than dorsal inactivation. The results from these studies are summarised in Table 2.

	Manipulation	Spatial Information	Field size	Stability	Peak firing rate	Theta power	Other observations
Miller & Best 1980	EC lesion (mainly MEC)	↓ *					Place fields not anchored to extramaze cues *robustness rather than SI
Brun *et al.* 2007	MEC LIII lesion	↓	↑	↓	–		
Van Cauter *et al.* 2008	MEC lesion	–	↓	↓	↓		Decrease in following rotating object cues. Increased remapping following cue removal
Brandon *et al.* 2014	MS inactivation Block grid firing	–	↑ *	–	↓	↓	Remapping occurred normally * during first 20 min only
Hales *et al.* 2014, Schlesiger *et al.* 2015	MEC lesion	↓ *	↑	↓	↓	↓	*due to drifting place fields rather than wider place fields
Ormond *et al.* 2015	Dorsal or Ventral MEC inactivation	↓	↑		↓	↓	

Table 2: Summary of previous MEC manipulation studies (↓ reduced, ↑ increased, – unchanged)

1.3.6 Summary and future work

Generally it appears that MEC lesions reduce in field firing rate, and lower the spatial precision and stability of place cells. However, even complete lesions of the MEC do not abolish place field firing in the MEC, suggesting that other inputs to the hippocampus contribute enough spatial information to allow place field formation. The decrease in spatial precision following MEC lesions, suggests a role for the MEC in refining or stabilising place fields. In addition the nature of the spatial code in MEC might allow place cell stabilisation via path integration, but this has not been directly

tested. The result that following MEC lesions place fields rotate with a maze rather than remaining anchored to the room suggests an inability to use distal landmarks for orientation, and together with result that place cells remap after the removal of object cues, suggests an overdependence of place cells upon local cues. These results suggest the hypothesis that the MEC allows the stabilisation of hippocampal place cells relative to distal landmarks, but is not required for their stabilisation relative to proximal landmarks. This hypothesis is supported by previous behavioural data from watermaze tasks showing that MEC lesions impair navigation based on extramaze cues but not intramaze cues, however electrophysiological data supporting this is incomplete. This hypothesis will be investigated in Chapter 4 of this thesis.

1.4 Aims of this thesis

Hippocampal place cells encode an animal's current location in an environment (O'Keefe and Dostrovsky 1971). Each place cell shows high firing rate in one or more small areas of the environment known as place fields. These place fields are maintained in a stable location using a combination of external landmarks (visual, olfactory or tactile cues) and internal self-motion information. Place cells are found throughout the hippocampus but are most commonly recorded from CA1 which is the final step in the hippocampal circuit. It is known that the firing rate of CA1 place cells can become modulated by additional features of a task such as the destination, or current trajectory when animals are performing navigational tasks with stereotyped trajectories. The aim of this thesis is to investigate several of the areas which project to CA1 or to the hippocampus in general and look at their different contributions to basic place field properties, trajectory-dependent activity, or spatial memory and navigation.

Experiment one will compare the place cell activity in CA1 with the activity of place cells in the upstream hippocampal area CA3. Trajectory-dependent activity is known to develop as animals learn a task, but the origin of the activity has not been established. Recently it has been suggested that trajectory encoding may originate in the mPFC and be passed on to CA1 place cells directly, bypassing place cells in upstream hippocampal areas such as CA3 (Ito *et al.* 2015). If this were the case, it would be expected that trajectory-dependent activity would be much less prevalent in CA3 place cells and would develop later in CA3 than in CA1. The aim of this experiment is to establish whether trajectory-dependent activity is present in CA3 place cells as rats perform a hippocampus-dependent spatial task. The extent and timepoint at which such activity develops will be compared between CA3 and CA1 place cells in order to answer this question.

Experiment two aims to look at the behavioural effects of removing the nucleus reuniens, a brain area suggested to provide trajectory information to the hippocampus.

Since the occurrence of trajectory-dependent activity has been shown to coincide with learning a spatial task, it is possible that removing a source of such information might impair learning or performance of the task. The aim of this experiment was to investigate whether lesions of the nucleus reuniens would cause deficits in acquisition or performance of a hippocampus-dependent task in which trajectory-dependent activity is known to coincide with learning task rules. It has also been proposed that the nucleus reuniens is only necessary when tasks require mPFC to hippocampus communication; for example when switching to an allocentric navigational strategy. A secondary aim of experiment two is to investigate this by comparing performance on tasks which might be expected to require mPFC-hippocampus interactions with tasks which would not be expected to involve communication between these brain areas.

The final experiment investigates the contribution of one of the main spatial inputs to the hippocampus. The medial entorhinal cortex provides one of the most precisely spatially modulated inputs to hippocampal place cells and was thought to be necessary for place field production. However several studies have shown that CA1 place cell activity appears relatively unchanged following MEC lesions. Place fields are still present but show mild reductions in stability and precision. The aim of this experiment is to investigate whether lesions to the MEC change the types of landmarks to which place cells are oriented. It is hypothesized that lesions of the MEC will impair the use of distal visual cues for place field orientation in a circular environment, but that the use of proximal cues will be unimpaired.

2 Trajectory dependent activity in CA3 place cells

2.1 Introduction

Trajectory dependent activity is a feature of place cell activity which has been observed in several different labs on several different tasks. It was first observed by Frank *et al*. (2000) on a W-maze alternation task, and by Wood *et al*. (2000) in a continuous alternation task on a T-maze. In both of these experiments, place cells with fields in the central stem of the maze showed either rate modulation or a shift in field location depending on the previous or upcoming path of the animal even though the external cues and immediate behaviour of the animal were unchanged. This suggested that place cell activity could be modulated not only by the animal's current location but also by future or past locations. This activity could allow the disambiguation of different overlapping trajectories through the same place, which might be useful for learning or performing spatial memory tasks.

One important question that remains to be answered is where this trajectory dependent activity originates. Frank *et al*. (2000) observed trajectory dependent activity in the CA1 region of the hippocampus and both the deep and superficial layers of the entorhinal cortex. This was also seen by Lipton *et al*. (2007) who found not only that trajectory dependent activity occurs in the dorsocaudal MEC, but that a greater proportion of cells in the MEC showed trajectory dependent activity than cells in CA1. They suggest therefore, that trajectory dependent activity originates not in CA1, but rather in the entorhinal cortex or perhaps even earlier. However, this is not necessarily the case. As the hippocampus and entorhinal cortex form a circuit, trajectory information could be integrated with spatial information at any point in the circuit and could then be passed on to the rest of the circuit. Alternatively, each area in the hippocampal-entorhinal circuit might be encoding space and trajectory in subtly different ways, resulting in different proportions of cells showing each kind of activity depending on the demands of the task. For example, CA3 contains many recurrent connections which might allow associations between place cells and therefore potentially locations. Whereas CA1 receives inputs from multiple areas within and outside the hippocampus and therefore the place cell representation (and potentially

also trajectory-dependent activity) might represent the combination of inputs from within and outside the hippocampus.

Since we know that both the MEC and CA1 show trajectory dependent activity the obvious intermediate step is CA3. Research by Johnson and Redish (2007) and Cabral *et al.* (2014b) suggests that CA3 may be involved in processing and storing trajectories as sequences of connected places or actions. Johnson and Redish showed that multiple sequences of CA3 place cells fire in sequence ahead of an animal's actual location while it is paused at a decision point in a maze. Cabral *et al.* used a starmaze task which could be solved either by performing a sequence of body turns, or by navigating to a place allocentrically. They inferred from the frequency of gamma oscillations in CA1 that the CA3 input was driving CA1 activity during sequence based trials and when the place cell map was representing the animal's position within a sequence rather than its allocentric location. If CA3 is involved in processing sequences of body turns or places then it is possible that as a task is learned, place cells within a trajectory might change their firing rate entirely or 'rate-remap' so that they fired more when activated in a sequence within one trajectory but not otherwise.

This idea has been tested in two different experiments with contradictory results. Bahar and Shapiro (2012) tested whether CA3 cells showed trajectory dependent activity in a plus maze task. They recorded from place cells within CA3 and CA1 while animals solved different tasks, either an allocentric place task (with serial reversals), a switch task in which the reward locations changed, or an altered environment in which all the cues changed. They found that approximately 50% of place cells in both CA1 and CA3 showed trajectory dependent activity in the allocentric task. This suggests that trajectory dependent activity is found in CA3 and is similar to that seen in CA1.

However recently, Ito *et al*. (2015) recorded from place cells in both CA1 and CA3 while animals performed a continuous spatial alternation task. This was similar to the task used in Wood *et al*. 2000. Surprisingly they found that place cells in CA3 showed far less trajectory dependent activity than they recorded in CA1. In addition they found that removing the input from the nucleus reuniens, which projects directly to CA1 but

not CA3, reduced the level of trajectory dependent activity in CA1 down to the level seen in CA3. Although this seems contradictory to the previous result from Bahar and Shapiro, one way to reconcile the two results may be that the demands of the tasks used are different. The continuous alternation task used by Ito *et al*. (in which no CA3 trajectory dependent activity was observed) is not a hippocampus-dependent task (Ainge *et al*. 2007b), whereas the plus maze place learning task, (in which trajectory dependent activity was observed in CA3 by Bahar *et al*.) is dependent upon the hippocampus (Packard and McGaugh 1996, Shapiro *et al.* 2006). This might suggest that the brain areas involved in generating and displaying trajectory dependent activity vary based on the various demands of the spatial task. If this is the case, it might be expected that in a hippocampus-dependent task, animals would show trajectory dependent activity throughout the hippocampus rather than just in CA1.

To test this we used the double-Y maze serial-reversal task. Ainge *et al*. first developed this task in 2007. The authors found that in a maze which contains a start box and several common alleyways which are common to all or a subset of the possible trajectories, CA1 place cells in these common areas typically fire in a trajectory dependent manner. Importantly, this task is hippocampus-dependent; animals with hippocampal lesions are unable to learn the task (Stevenson 2011) and if they are pretrained on the task before hippocampal lesions, they still show deficits in switching between reward locations (Ainge *et al* 2007a). In addition, a study into the time-profile of when trajectory dependent activity develops relative to acquisition of the task revealed that place cell activity is not modulated by trajectory during initial days of training, or during random foraging on the maze, but only becomes modulated by trajectory as the serial-reversal task is learned. This suggests that in this task, the appearance of trajectory dependent activity may represent learning the task rules (Huang 2010, Stevenson 2011). This task then, would be ideal to look at whether trajectory dependent place cell activity in hippocampus-dependent tasks originates or at least occurs in CA3, rather than originating in CA1 based on inputs from the nucleus reuniens or elsewhere.

The aim of this experiment is to compare the activity of place cells in CA3 to that seen in CA1 in the double-Y maze serial-reversal task, in order to further explore the origin of trajectory dependent activity within the hippocampal formation. This will be accomplished by recording from place cells in both areas simultaneously as rats learn the serial-reversal task on the double-Y-maze. If trajectory dependent activity develops at the same time point in CA3 and CA1, and is seen to the same extent in both areas, then this would suggest that trajectory dependent activity does not originate in CA1 and may be inherited from CA3 rather than being dependent upon the inputs from the nucleus reuniens. On the other hand, if less trajectory dependent activity is seen in CA3 then this would suggest that trajectory dependent activity either originates in CA1 or another brain area, such as the nucleus reuniens which does not project into the hippocampus via CA3.

2.2 Methods

2.2.1 Subjects

The experiment was carried out using 10 male Lister-Hooded Rats. An additional 10 rats underwent electrode implantation but no single-units were identified during screening so they were not included in the experiment. Rats weighed between 400-600 g at the time of surgery. Following surgery, rats were individually housed to prevent damage to microdrives. Rats were kept in a 12 hr light/dark cycle and behavioural training occurred during the light phase of the cycle. Rats had free access to water in the home cage. Following recovery from surgery, food was restricted to maintain them at or above 85% of their free-feeding weight. All procedures complied with the Animals (Scientific Procedures) Act 1986.

2.2.2 Micro-drive implants

Microdrives were based on Kubie's tripod design (1984) which was modified to allow two four-tetrode drives to be implanted onto one animal. Microdrives consisted of a tripod of drive screws, the electrode bundle, an 18 pin MillMax connector (MillMax, NY) and a dental cement base (Figure 1a).

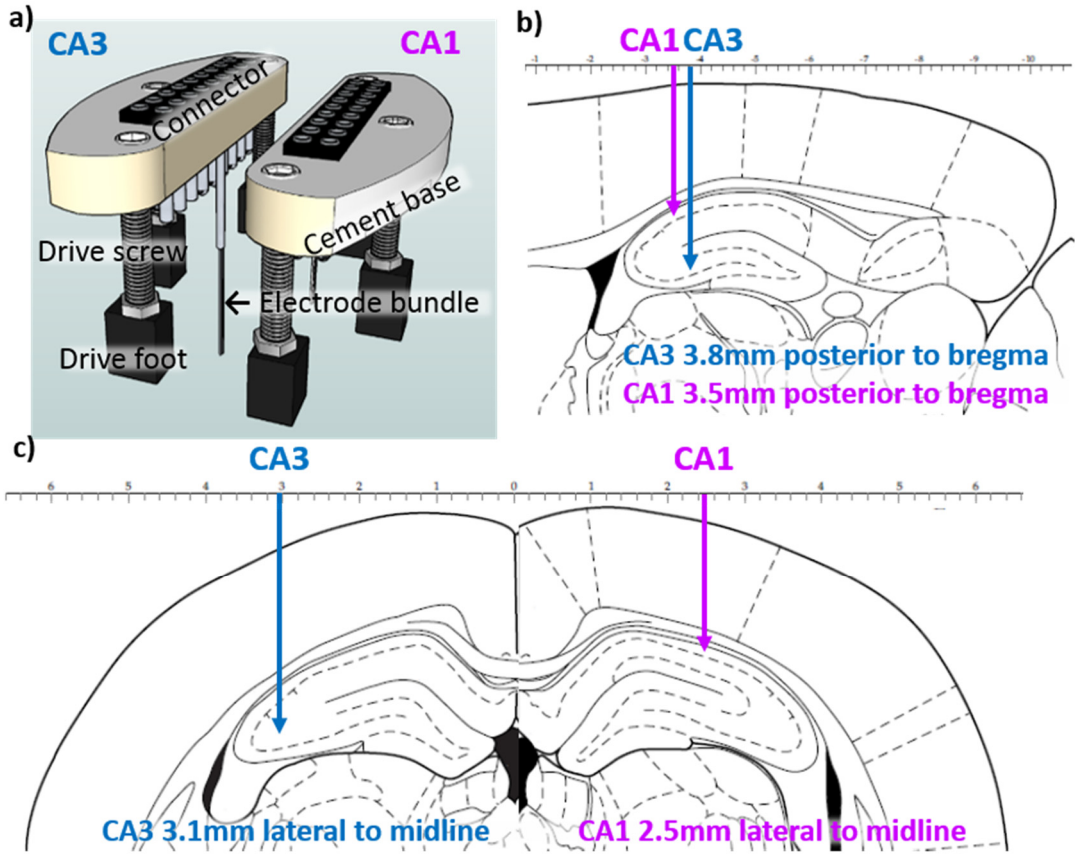


Figure 1: a) Bilateral microdrive Implant b) locations of the CA1 and CA3 electrodes in the sagittal plane c) locations of the CA1 and CA3 electrodes in the coronal plane

The drive tripod was constructed from 3 stainless steel 80 TPI drive screws (Precision Technology Supplies, UK) 3 stainless steel 80 TPI nuts (Precision Technology Supplies, UK) and three tapped Amphenol sockets (Amphenol Ltd, UK). The nuts were superglued to the top of the Amphenol sockets to form 'feet' into which the drive screws could be lowered. The drive screws were connected to the MillMax connector with dental cement (Simplex rapid acrylic denture polymer, Associated Dental Products Ltd, UK) so that they could be turned to lower the drive.

Tetrodes were constructed from 17 µm HML-coated platinum (90%)-iridium(10%) wire (California Fine Wire, CA). 4 lengths of wire were twisted and heat-annealed together using a heat gun at 240°C for ~6 seconds to form each tetrode. 4 tetrodes were then loaded together into a 24 gauge stainless steel support cannula (Small Parts Inc, Miramar, FL) and mounted onto the MillMax connecter.

Both the support cannula and an additional 30 gauge insulated ground wire (Vishay Precision Group, Germany) were soldered to the ground pins of the MillMax connector. The electrode was then stabilised within the cannula using super-glue. After the insulation was removed from ~3 mm end of each wire of a tetrode, they were connected individually to one pin of the MillMax connector using silver conductive paint (Electrolube, UK). The connections were then insulated and stabilized by the application of non-conductive varnish (Spray Acrylic, Electrolube, UK) followed by nail-varnish.

Shortly before surgery, the electrode bundle was trimmed using ceramic scissors (Fine Science Tools, Germany) under a microscope to length 3-5 mm longer than the base of the feet. The tetrode was then placed within a solution of gold (Non-Cyanide Gold plating solution, Neuralynx, MT) and connected to a DC power supply. The tetrode tips were first cleaned by passing four 1-second pulses of 4 μA through them with the tetrodes as the anode. The tetrode tips were then gold-plated using direct current pulses of 2 μA of 1 second duration with the tetrode as the cathode, until the impedance dropped to 150 kΩ. A protective 18 gauge outer cannula was then placed around the support cannula and electrode bundle and held in place using sterile Vaseline.

Seven of the rats were implanted with one of the drives into CA3 in the right hemisphere and the other into CA1 in the left hemisphere. Three additional rats were implanted with unilateral 8 tetrode microdrives (2 into CA1, 1 into CA3). These drives were identical to the drives described above except that they had double the number of channels. All eight tetrodes were loaded into one cannula.

2.2.3 Surgical procedures

Rats were anaesthetized using isofluorane gas (Abbott Laboratories, IL), and in addition, 0.08 ml/kg bodyweight small animal Rimadyl (Pfitzer, UK) was administered subcutaneously at the start of surgery as analgesia. Subcutaneous injections of isotonic saline and glucose solution were administered at the start of surgery and later as needed to maintain hydration. The eyes were covered throughout surgery with hydrating eye-gel (Viscotears, TX). The rat was placed on a thermostatic

heat blanket and covered with a sterile drape. The rat was then fixed into a stereotaxic frame (Kopf, CA) using a bite bar, nose cone and two non-traumatic ear-bars. The skull was exposed via a midline scalp incision, and 6-8 self-tapping skull screws (Fine Science Tools, Germany) were inserted into pre-drilled holes in the skull.

For the bilateral microdrives (7 rats), two holes ~1 mm in diameter were then drilled; one above CA3 in the right hemisphere and one above CA1 in the left hemisphere. Dura was pierced and the electrodes were lowered into position. Coordinates for the CA3 electrode were, relative to Bregma: 3.8 mm posterior, 3.1 mm lateral and 3 mm below dura (Figure 1b&c). Coordinates for the CA1 electrode were, relative to Bregma: 3.5 mm posterior, 2.5 mm lateral and 1.8 mm below dura (Figure 1b&c). The protective cannulae were lowered into position around the electrodes above the skull, and sterile Vaseline was used to ensure the join was sealed. The ground wires for each drive were connected to separate skull screws using silver paint. Dental cement (Simplex rapid acrylic denture polymer, Associated Dental Products Ltd, UK) was then used to attach the drive feet and protective cannulae to the skull screws.

For the unilateral microdrives (3 rats), one hole was drilled either above CA1 in the left hemisphere (2 rats), or above CA3 in the right hemisphere (1 rat). The implantation coordinates were the same as those described for the bilateral implants and the procedure for implantation, grounding, and cementing were identical.

The implants were then surrounded with electrical tape to protect them. Rats were placed in recovery cages on a heat bench at 30°C until they fully regained consciousness and then for a further hour of recovery. Rats were given 10 days of recovery on free food before recording or behavioural training, during which time all rats regained their presurgery weight.

Apparatus

The screening environment consisted of a large plastic flowerpot 1m in diameter with a wooden floor painted black. A high-contrast cue card was attached to the inside of the flowerpot.

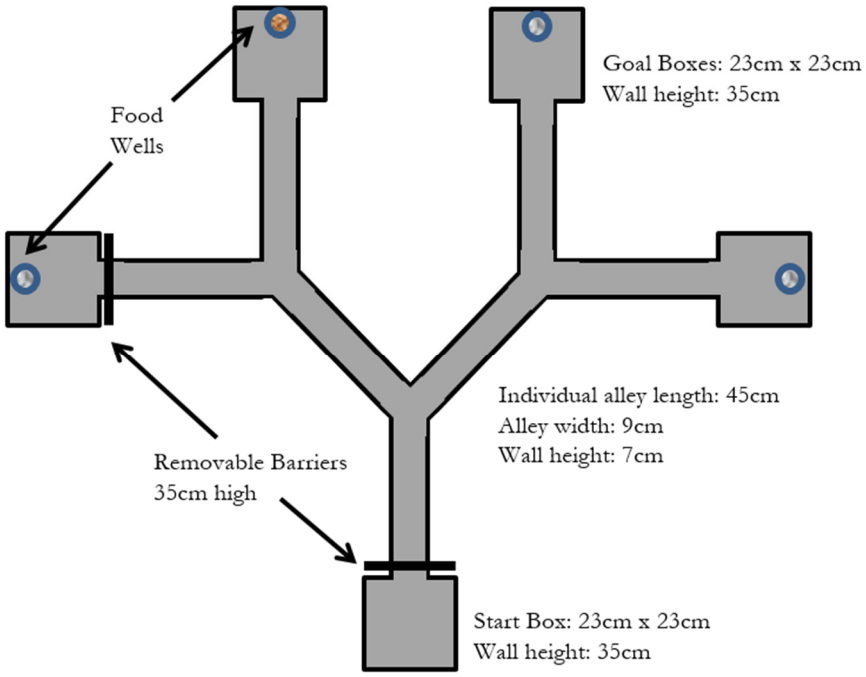


Figure 2: Plan of double Y-maze apparatus with dimensions

The double Y maze was built out of wood and painted with black paint. It consisted of a start box and 4 identical goal boxes connected by a double-Y shaped alleyway as shown in Figure 2. All the boxes were square and measured 23 cm across and 35 cm high. The alleyways were 9 m wide with walls 7 cm high. Each straight alley section was 45 cm long giving a total length from start to goal of ~135 cm. Barriers 35 cm high were used to prevent animals from leaving the start box and goal boxes between trials. Each goal box contained a food well which either contained Coco Pops (Kellogg's, UK) or was empty. Beneath a metal grill in each well were additional inaccessible coco-pops to ensure that all food wells smelled of the food reward.

2.2.4 Electrophysiological recording

Following 10 days recovery after surgery, rats were connected to a 32-channel recording system (Axona Ltd, UK) via a headstage amplifier, commutator, and pre-amplifier. Rats were then placed in the screening environment and were allowed to forage freely for scattered coco-pops. The signal was amplified, filtered with a bandpass filter at 600-6000 Hz and potential single-units were identified by eye on an oscilloscope trace using DACQ software (Axona Ltd, UK). If single-units were

observed, a trigger was placed at an appropriate amplitude threshold for each channel to detect unit activity while minimizing collection of noise spikes. One channel from each drive was used to record a local field potential using a lowpass filter at 300 Hz and a notch filter at 50 Hz. A camera placed above the environment was used in conjunction with an infrared LED on the rat's head in order to capture position coordinates during all recordings. Screening recordings were typically 5-10 minutes long. Spike data were analysed as described later and if no pyramidal neurons were present, the electrode was advanced by 40-80 μm into the brain. Screening was performed once or twice per day until pyramidal neurons were found. Once pyramidal neurons were found, animals were immediately trained on the behavioural task and all sessions were recorded in the same way as the screening sessions.

2.2.5 Behavioural task

Following identification of place cells, rats were trained on a serial reversal task on the double Y-maze. In every trial the rat was placed into the start box where he was confined for ~5 seconds using the moveable barrier. The barrier was then removed and the rat was allowed to explore the maze until he entered one of the goal boxes. If the rat entered an unrewarded box he was blocked in for ~5 seconds using a barrier, before being picked up and placed back in the start box. Upon entering the rewarded box, the rat was allowed to consume the reward for ~5 seconds before being placed back in the start box to start the next trial. The maze was wiped down with soapy water between trials to remove odour cues. The rat was given unlimited trials until he entered the rewarded box. Following the first correct trial to a rewarded box he was given 10 more trials in which the rewarded box remained the same. Following this, the rewarded box was moved to a new location to start the next block of trials. See Figure 3 for a break-down of an example day's training session. This was repeated for a total of four blocks until each of the four goal boxes had been rewarded once. The order in which the boxes were rewarded was varied randomly each day. Rats were trained and on this task for up to 13 days or until place cell activity was no longer detectable. Goal box choice, and correct goal box was recorded for every trial. Single-unit activity was recorded during every training session following the same procedures as for screening sessions.

2.2.6 Behavioural data analysis

Performance and acquisition of the task was measured in two ways. Firstly, the number of trials taken to find a new reward location was counted for each block of trials (Switching Trials (Figure 3b)). Secondly the number of correct returning trials out of 10 after finding the reward location (% Correct Returns (Figure 3c)). These performance measures were averaged across all blocks in one day. A significance threshold of $p<0.05$ was used throughout this thesis.

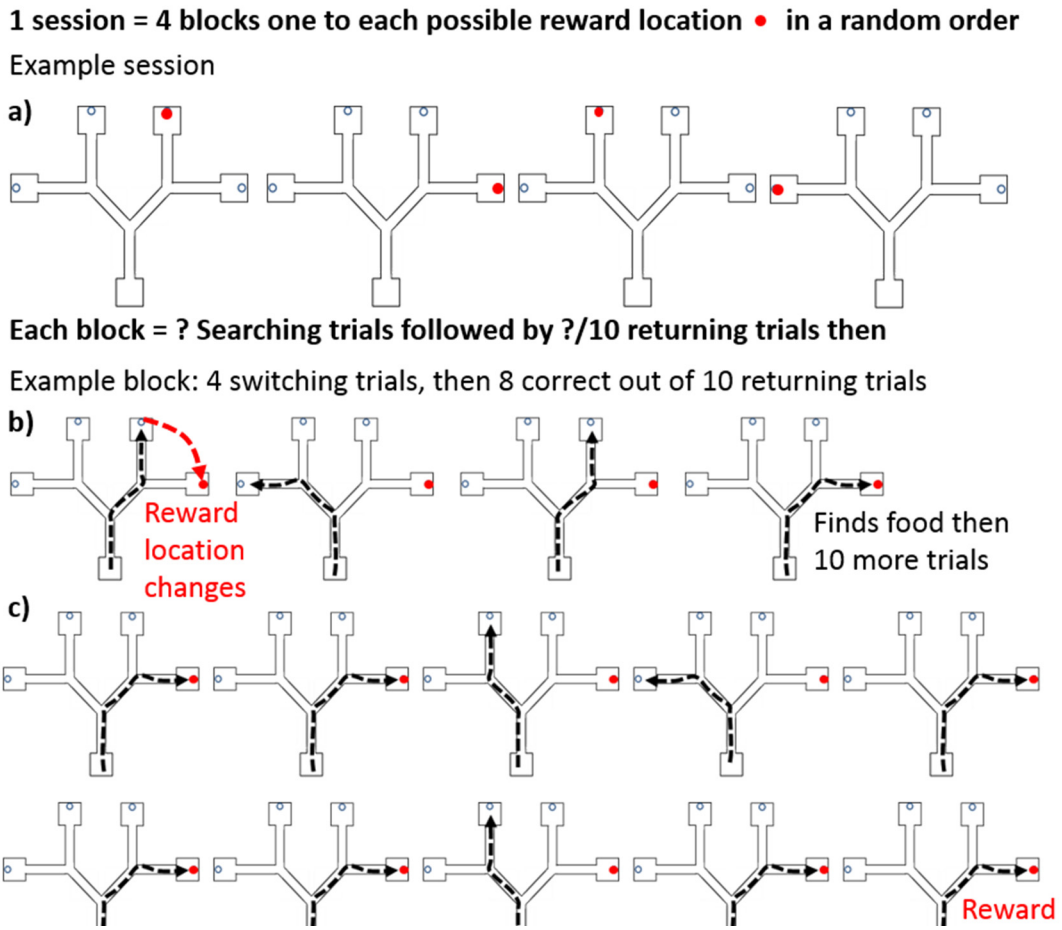


Figure 3: Plan of one session of training a) four blocks one to each possible reward location b) example with 4 switching trials c) example returning trials with a performance of 80% correct returns

2.2.7 Electrophysiological data analysis

Following data collection by Dacq-USB (Axona Ltd, UK), data files were initially analysed using a custom Matlab script and a clustering algorithm (KlustaKwik, Kadir *et al*. 2014). Clusters identified by the algorithm were then visualised using Klusters,

(a GUI developed by Hazan *et al*. (2006)) so that noise clusters could be deleted and incorrect clustering could be fixed. A further Matlab script was then used to generate firing-rate maps for each cluster, plot average waveforms for each channel of the tetrode, and calculate overall firing rate, waveform width and spatial information content. The firing rate map was constructed using an algorithm adapted from Leutgeb *et al.* (2007a). The entire area explored by the animal was divided into pixels 2.5x2.5 cm in size. Firing rates were not calculated for a pixel if the animal never came within 5 cm of the centre of the pixel. The average firing rate for each pixel was calculated using every spike recorded, but weighted using a Gaussian kernel such that spikes close to the centre of the pixel had the most effect on the resultant firing rate. The firing rate (FR) of a given pixel with centre position *x* was calculated as follows:

$$\mathrm{FR}(x) = \frac{\sum_{i=1}^{n} g\left(\frac{S_{i-x}}{h}\right)}{\int_0^T g\left(\frac{y(t)-x}{h}\right) dt}$$

where *i* is every spike from 1 to the total n of spikes. S*i* is the position of every spike, [0 T] is the total time of the recording period, *y(t)* represents the animal's position at time t, and h is the smoothing factor of 2.5 cm, and *g* represents a Gaussian kernel with equation: $g(x) = exp\left(\frac{-x^2}{2}\right)$

The overall firing rate was calculated as the total number of spikes within a session divided by the length of the session.

Spatial information content (SI) was calculated using the following equation: $\mathrm{SI} = \sum_i P_i \left(\frac{R_i}{R}\right) ln\left(\frac{R_i}{R}\right)$, where *i* is the bin number in the firing rate map, P_i is the probability that bin *i* is occupied, R_i is the mean firing rate in bin *i* and R is the overall mean firing rate. Spatial information content is a measure of how much information about location is carried by one spike (Skaggs *et al.* 1993).

Clusters were identified as place cells if they had a firing rate between 0.1-5Hz, a waveform width >250uS and if the spatial information content >0.5bits/spike. Clusters which did not meet these criteria were excluded from further analyses.

Another Matlab script separated the recording session into trials by comparing the position data to the locations of the start box and reward boxes to identify trial start and end times. The spike and position data were than split up based on these trial start and end times and the destination of each trial was identified as one of the four goal boxes. To analyse trajectory-dependent activity, the trials were sorted into the four possible trajectory destinations. The maze was subdivided into the four areas in which trajectory dependent activity could take place: the start box, the initial central alley, the left and right alleys following the initial Y junction (Figure 4). The firing rate in each of these areas was then calculated for each individual trial as the number of spikes within the area divided by the total time spent within the maze area, and an ANOVA was used to determine whether firing rate differed significantly ($p<0.05$) based on the trial's destination.

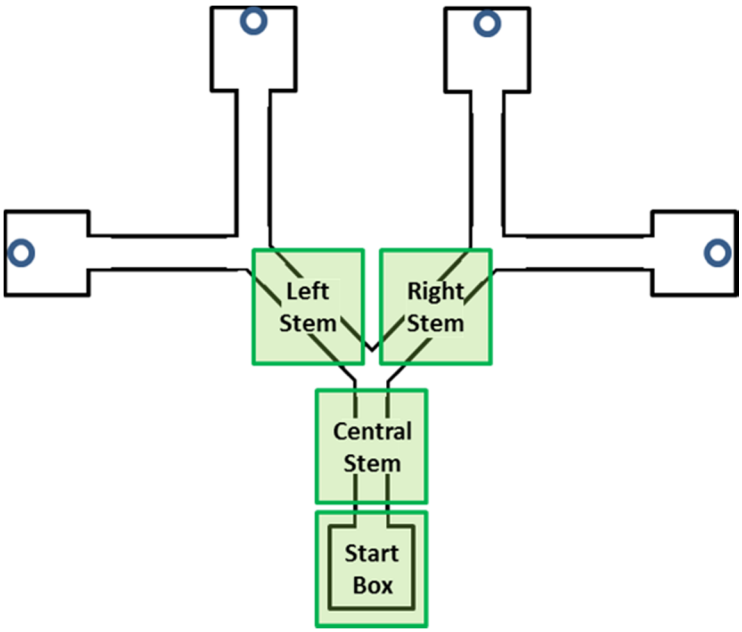


Figure 4: Maze areas used for identification of trajectory dependent activity. Mean firing rates were calculated as the total number of spikes within each box divided by the total time spent within each box during each individual trajectory.

In addition the max rate change was calculated; the firing rate in each maze area was calculated for each trajectory as described above, and an average taken across trials to each destination. The destination with the highest average firing rate within the area and the destination with the lowest average firing rate within the maze area were used

to calculate the max rate change as given by the following equation: $Max\ rate\ change = \frac{\overline{FR}_{Max} - \overline{FR}_{Min}}{\overline{FR}_{Max}} * 100$

Where $\overline{FR}_{Max}$ is the highest trajectory type mean firing rate and $\overline{FR}_{Min}$ is the lowest trajectory type mean firing rate.

2.2.8 Histology

Following completion of data collection, rats were anaesthetized with isofluorane and given a lethal dose of sodium pentobarbitol (Euthatal, Meridal Animal Health, UK). The tissues were fixed by transcardial perfusion of 0.9% saline followed by 4% formalin. The brains were then extracted and stored at room temperature in 4% formalin. They were then flash-frozen and sectioned at 30 μm thickness with a cryostat-microtome. Sections were mounted on polysine slides (Thermo Scientific, UK), stained with 0.1% Cressyl Violet, and coverslipped in DPX (Sigma-Aldrich, UK). Sections were then examined under 10-20x magnification with a light-microscope to identify the deepest extent of each electrode track. These sections were then photographed using Image-Pro Plus (Media Cybernetics, USA).

2.3 Results

2.3.1 Task acquisition and performance

Data on task acquisition was collected from 8 animals. All animals began training on the task as soon as pyramidal cells had been identified. However, training was stopped if pyramidal cells were no longer apparent during the recordings. Consequently, some animals were not trained for long enough to observe them reach a plateau in performance. The data for five of the animals was complete for the first seven days of training and so their performance was assessed using a repeated measures ANOVA.

The performance was measured in two ways. Percent correct returns measures win-stay performance, and is calculated as the percentage of trials after finding the food for the first time in a new location in which the animal correctly returned to the food location, averaged across all blocks each day. The number of switch trials before the animal found each new goal location was counted and an average was taken across the

four blocks of the day. Switching trials is a measure of the lose-shift aspect of the task and is a count of how many trials the animal took to find the food in each new location.

Figure 5a shows the percentage correct returns for each animal. It is apparent that all animals showed a similar trajectory of learning despite the fact that some animals did not complete all days of training. To perform a repeated measures ANOVA each subject needs to be used on every day of training. Since some animals dropped out and did not complete all training days analysis was performed on the five animals who completed seven days of training. Figure 5b shows the overall average and SEM for all animals that completed each day of training in colour and the average for the five animals that completed seven days of training and were used for statistical analysis in black. The average for these animals is similar to the average for all animals and an RM-ANOVA was used to determine whether these animals showed learning over the first seven days. There was a significant effect of day [$F_{(6,24)}$=13.8; $p<0.001$] and a significant linear trend of day [$F_{(1,4)}$=20.0; $p=0.01$]. This confirms that animals did show learning during the first seven days of training. Looking at the averages and standard error of the mean for percent correct returns, it is appears that animals reached asymptotic performance at day 5 of learning, after which performance remained at around 77% correct for the rest of training. This suggests that learning the win-stay rule occurs during the first five days of training.

The number of switching trials was also analysed in the same way. Figure 5c displays the individual animal results and Figure 5d shows the overall average and SEM for all animals that completed each day of training and the five animals analysed statistically. There appears to be a trend towards a decrease in switching trials over days, however there was a lot of variability between animals (Figure 5c) and all animals did not show this trend. There was no significant effect of day on the number of switching errors [$F_{(6,24)}$=1.2; $p=0.35$].

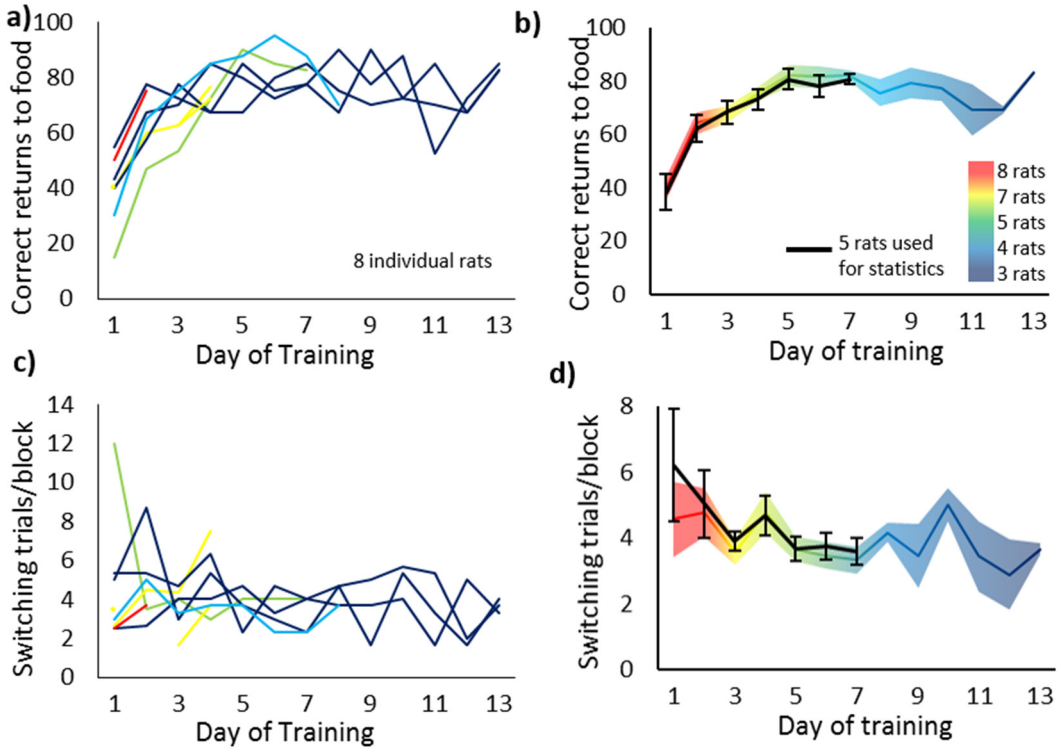


Figure 5: Performance during task learning a & b) Correct returns out of ten to rewarded goal box after the animal has found the reward location- averaged across all completed blocks. a) individual animal learning curves for correct returns to food b) coloured band shows the mean +/- SEM for all animals who completed a given day of training (colour shows how many animals are included at each stage), black line shows the mean +/- SEM from the five animals who completed seven days of training and were therefore used for statistical analysis of acquisition. The learning curve from the five animals is not greatly different from the learning curve when all animals are included. c & d) Number of trials taken to find the new reward location after the reward changed location – averaged across all completed switches. c) individual animal learning curves for switching trials d) coloured band shows the mean +/- SEM for all animals who completed a given day of training (colour shows how many animals are included at each stage), black line shows the mean +/- SEM from the five animals who completed seven days of training and were therefore used for statistical analysis of acquisition. The learning curve from the five animals is not greatly different from the learning curve when all animals are included except on the first day.

2.3.2 Pyramidal cell activity on the maze

Pyramidal cells were recorded from ten animals in total. Cells were identified in both CA3 and CA1 in four animals, in CA3 only in an additional four animals, and from CA1 only in two more animals. In total 383 cells in CA3 were identified as place cells and were active in one of the analysed areas, and 218 were identified in CA1. In addition a further 316 cells in CA3 and 210 CA1 cells were identified as place cells

without active place fields in the analysed areas of the maze. These cells were not included in further analysis.

2.3.3 Extent of trajectory dependent activity

Since learning had occurred by day five of training, days 5 onwards were considered as post-learning days. The data from these days was pooled to compare the overall differences in trajectory dependent activity between CA3 and CA1 once animals had learned the task. After removing the first four days of data, 286 CA3 cells and 84 CA1 cells were active with a firing rate of 1 Hz or more in one or more of the common areas of the maze. Table 1 shows the number of cells recorded from each brain area which were active in each of the analysed maze areas.

	Start Box	Central Stem	Left Stem	Right Stem	Any Area
CA3	152	123	136	92	286
CA1	54	43	38	56	84

Table 1: Number of cells recorded in each area after task performance had reached asymptote

The extent of trajectory dependent activity was analysed in two different ways. One method involved running an ANOVA on the firing rates from each individual trajectory through each area in which the cells was active (FR>1Hz) to look for a significant effect of trajectory destination in order to categorise every cell as either showing significant trajectory dependent activity or not. Figures 6-8 display example cells recorded in CA3 from each of the four areas of the maze. The bar graph displays the firing rates in the area of interest for each trajectory type. Blue graphs indicate that the ANOVA result is significant ($p<0.05$) for that area of interest. Figure 9 shows example CA1 cells from each maze area.

Figures 6-9: Example CA3 cells active in the start box (Figure 6), central stem (Figure 7), side stems (Figure 8). Example CA1 cells (Figure 9). Column 1: Waveform. Column 2: Mean firing rate on each trajectory type +/- SEM (Blue indicates significant difference between destinations ANOVA). Max rate change is the percentage change between the highest and lowest bars on the graph. Column 3: Trajectories sorted based on destination (black), with locations of spikes (red), Column 4: Firing rate map for all trajectories to each destination

Figure 6: CA3 – Start Box

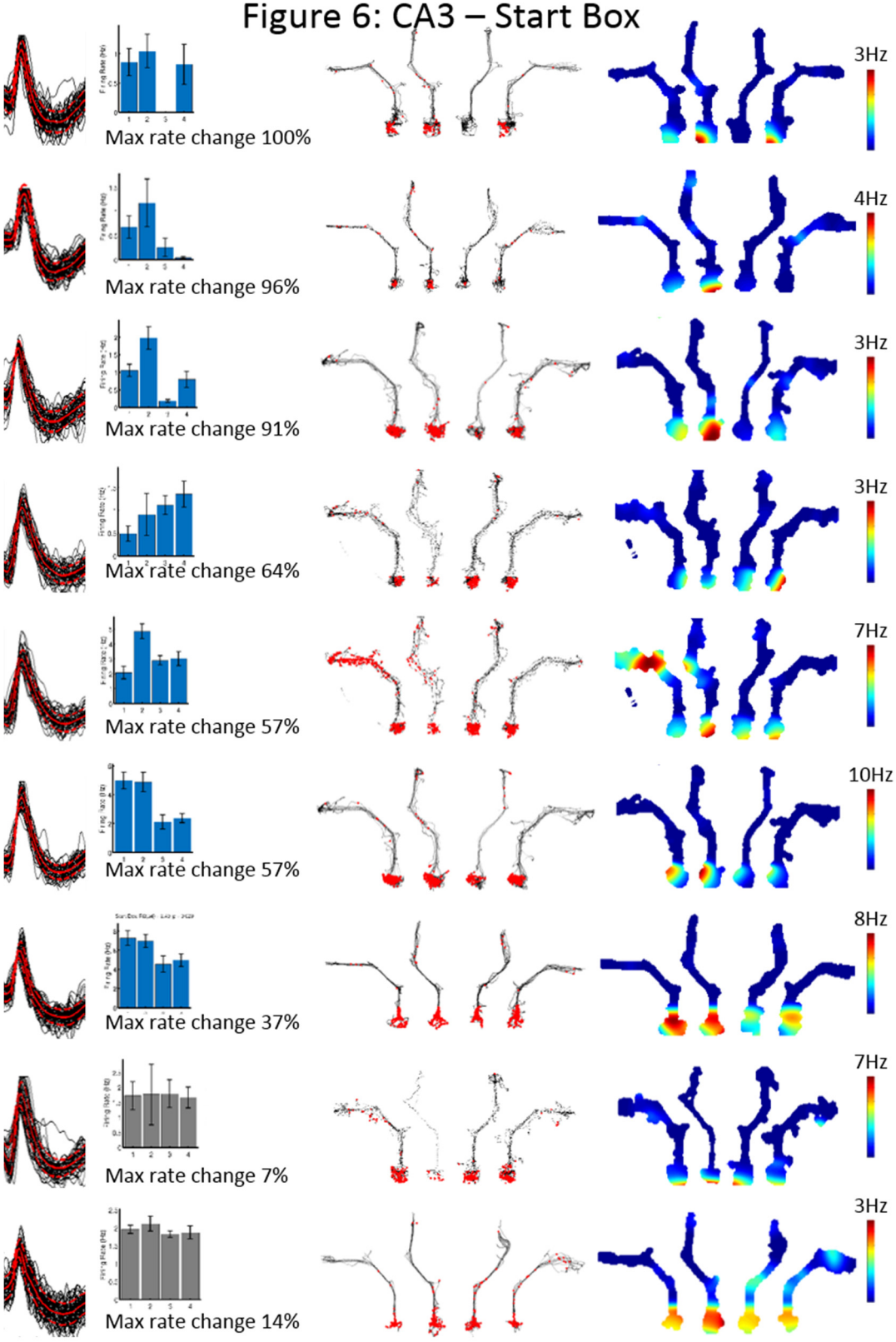


Figure 7: CA3 – Central Stem

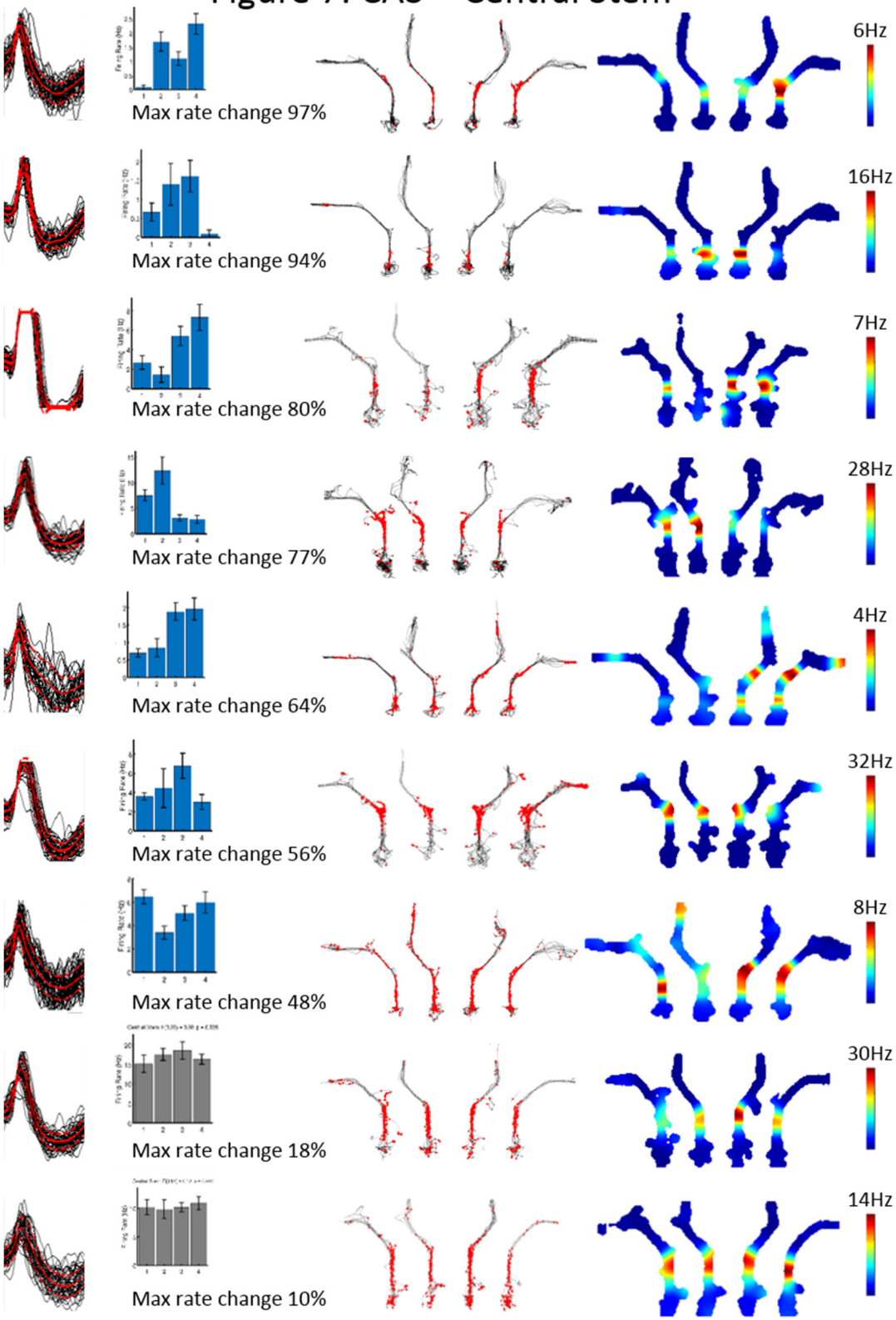
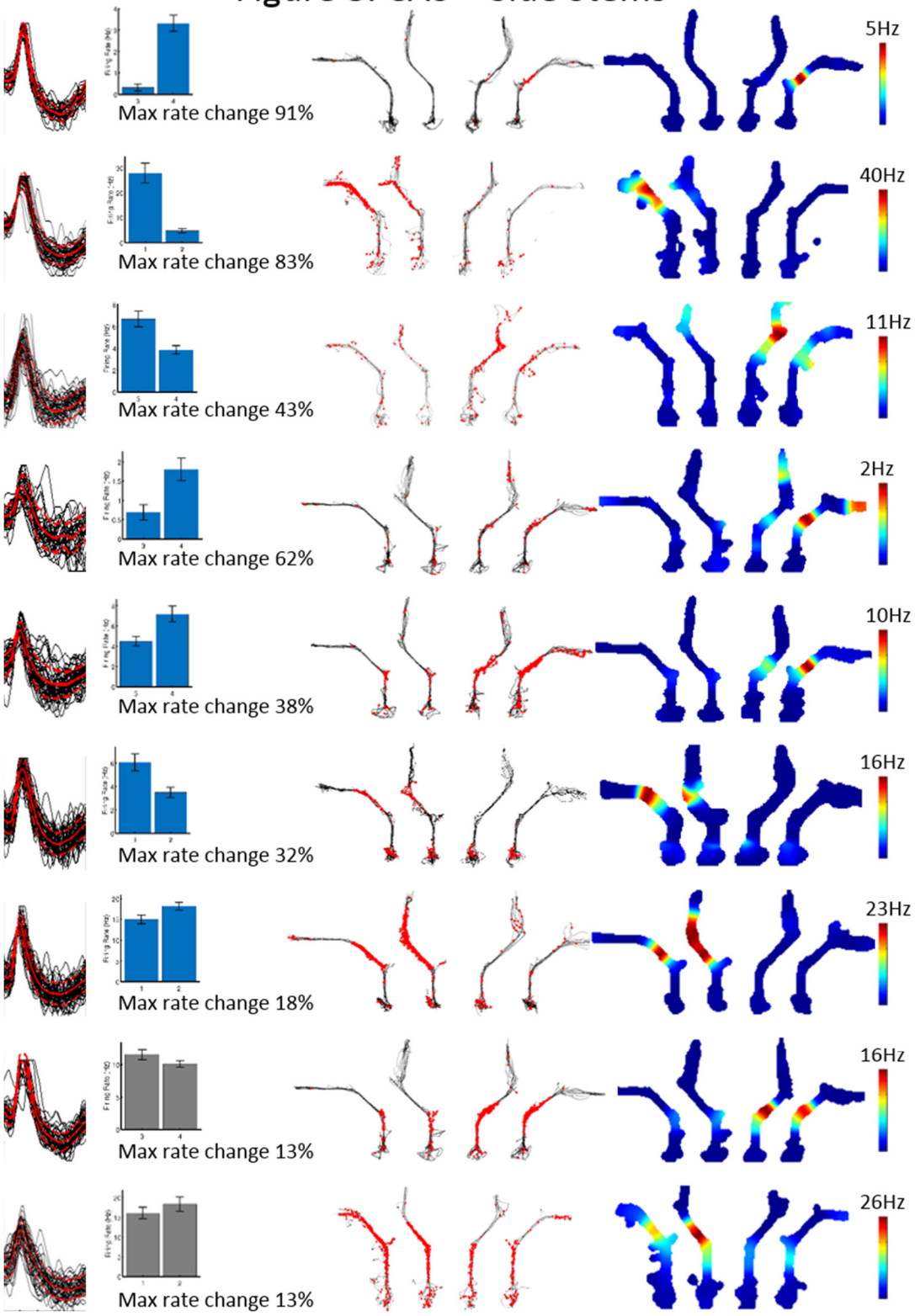


Figure 8: CA3 – Side Stems



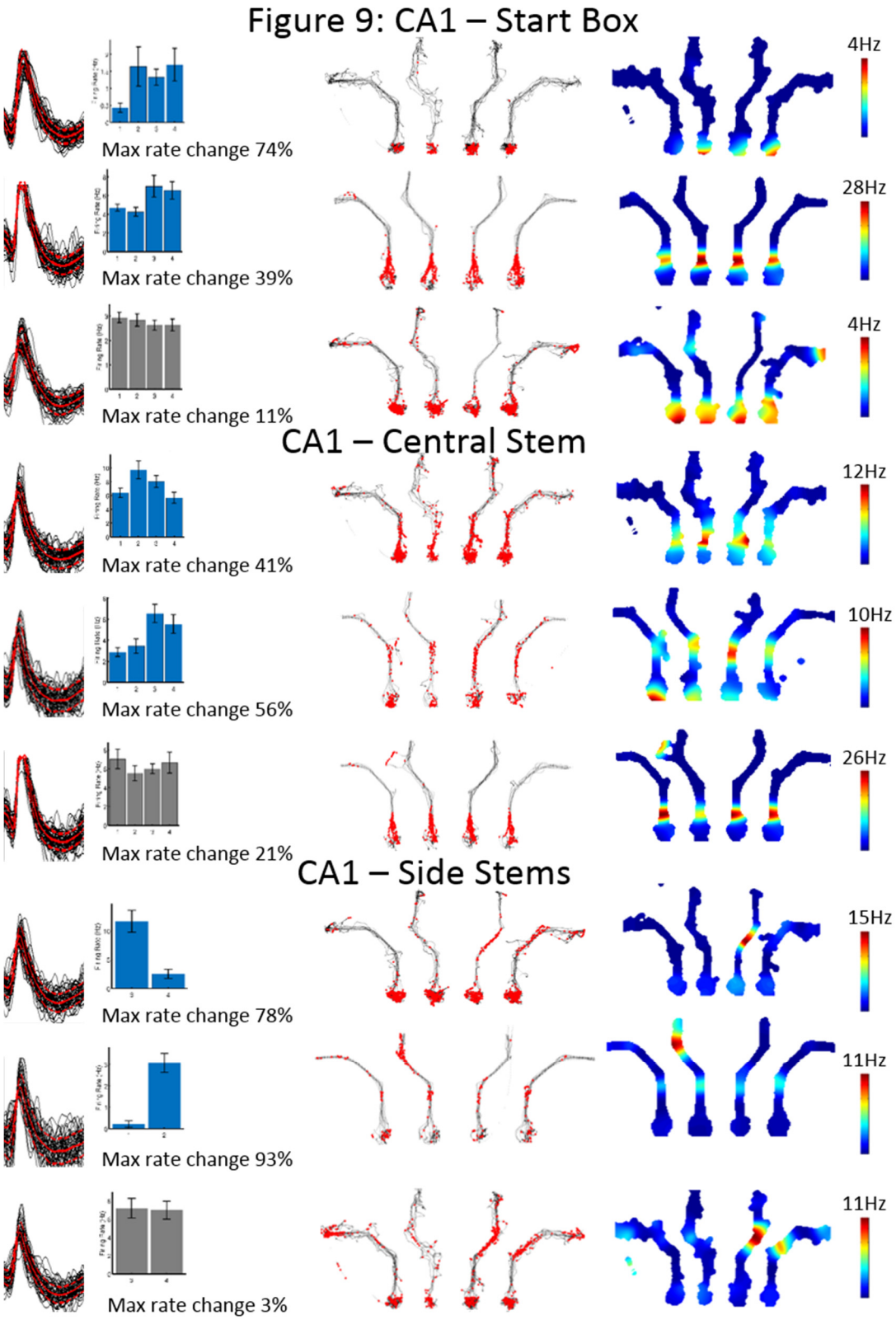


Figure 9: CA1 – Start Box
Max rate change 74%
4Hz
Max rate change 39%
28Hz
Max rate change 11%
4Hz
CA1 – Central Stem
Max rate change 41%
12Hz
Max rate change 56%
10Hz
Max rate change 21%
26Hz
CA1 – Side Stems
Max rate change 78%
15Hz
Max rate change 93%
11Hz
Max rate change 3%
11Hz

The other non-categorical method of analysing the amount of trajectory-dependent activity involved calculating the difference in mean firing rate between the trajectory with the highest firing rate and the trajectory type with the lowest firing rate for every cell with a highest firing rate of greater than 1Hz. This maximum rate change is displayed on Figures 6-9 and the example cells are arranged in order of decreasing max rate change. Maximum rate change ranged widely between very little change and 100% rate change. Figure 10a-d shows the distribution of maximum rate change for all cells active in each maze area, and the data is summarised as a box-whisker plot in Figure 10e. It is clear that the distributions do not vary much between the two brain areas, although slightly more CA3 cells than CA1 cells show 100% maximum rate changes between trajectory types in the start box and central stem. The maximum rate change for cells in CA3 and CA1 were compared with a Mann Whitney U test. No significant difference was found between the brain areas [$p>0.05$ for all maze areas (Start box $p=0.06$, Central stem $p=0.27$, Left stem $p=0.09$, Right stem $p=0.35$)].

Of the 286 CA3 cells active in the common parts of the maze, 122 cells (or 43%) showed significant firing rate changes between the different trajectory types (ANOVA $p<0.05$) Fewer CA1 cells were recorded over all, particularly after the initial days of training. However, 84 CA1 cells were active in the common parts of the maze, of which 55 cells (or 65%) showed a significant difference in firing rate dependent on the trajectory destination in at least one of the common areas of the maze (ANOVA $p<0.05$). This analysis was also performed for each of the four common areas individually and the results are displayed in Figure 10f.

A chi-square test was performed to test whether the overall proportion of cells which showed trajectory dependent activity was significantly different between brain areas. This analysis found no difference between the brain areas on the amount of trajectory dependent activity in the start box or the central stem but there was significantly more trajectory dependent activity in CA1 for place cells active in the side stems and overall.

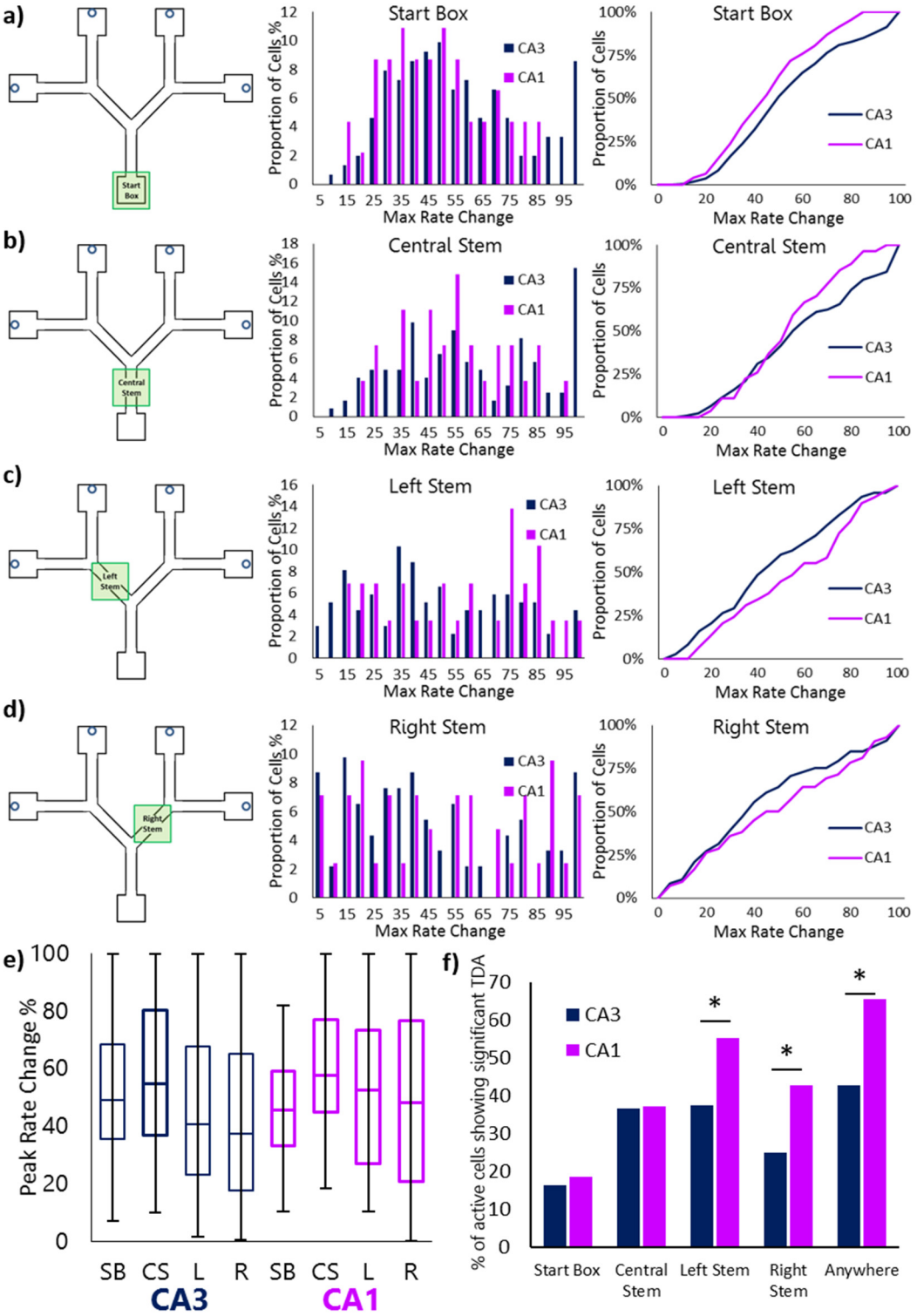


Figure 10: Extent of trajectory dependent activity a-d) left to right: maze area of analysis, distribution of maximum rate change for all cells active in the area, cumulative frequency distribution of maximum rate change for all cells active in the area. e) box-whisker summaries of the maximum firing rate change for each area f) proportion of active cells (FR>1Hz) in each maze area in CA3 blue and CA1 (magenta) that showed a statistically significant effect of trajectory destination on firing rate (Trajectory Dependent Activity). Asterisks show significant effect of brain area [Chi Square p<0.05].

2.3.4 Development of trajectory dependent activity during learning

To analyse the development of trajectory dependent activity, the proportion of cells which showed trajectory dependent activity were calculated for each animal on each day. Figure 11a&b shows the total number of cells active on the maze each day (grey) and the number of cells showing trajectory dependent activity (blue) in CA3 (Figure 11a) and CA1 (Figure 11b). It is obvious that the number of recorded cells decreases dramatically after day 7. Because of the limited sample sizes the data after this point may be variable, therefore statistical tests were only run on the data from the first seven days. Figure 11c shows the overall proportion of cells showing trajectory dependent activity on each day. The proportion of cells showing trajectory dependent activity generally increases over the first few days of task acquisition and there is little difference between the brain areas, at least during early days with high total cell numbers. Figure 11d shows the mean and SEM of the proportions from each animal. There appears to be a slightly higher proportion of trajectory dependent activity in CA1 than in CA3 although there is a high level of variability between animals as seen in the wide error bars. This is possibly because of the high variability in number of cells recorded on each day, and because on days when the proportion of trajectory dependent activity was calculated from a very small number of cells the result could be variable.

The proportion of cells showing trajectory dependent activity shows an overall trend of increasing over day. Analysis was performed with a univariate unbalanced ANOVA with both brain region and day as factors. When all sessions recorded over the first seven days (Figure 11d) were included in the analysis, there was both a significant effect of brain region [$F_{(1,61)}$=10.1; p=0.02] and of day [$F_{(6,61)}$=2.3; p=0.048], but no significant interaction between the two [$F_{(6,61)}$ =0.9; p=0.5]. Sessions in which only a few cells had been recorded often resulted in proportions of 0 or 100% trajectory dependent activity.

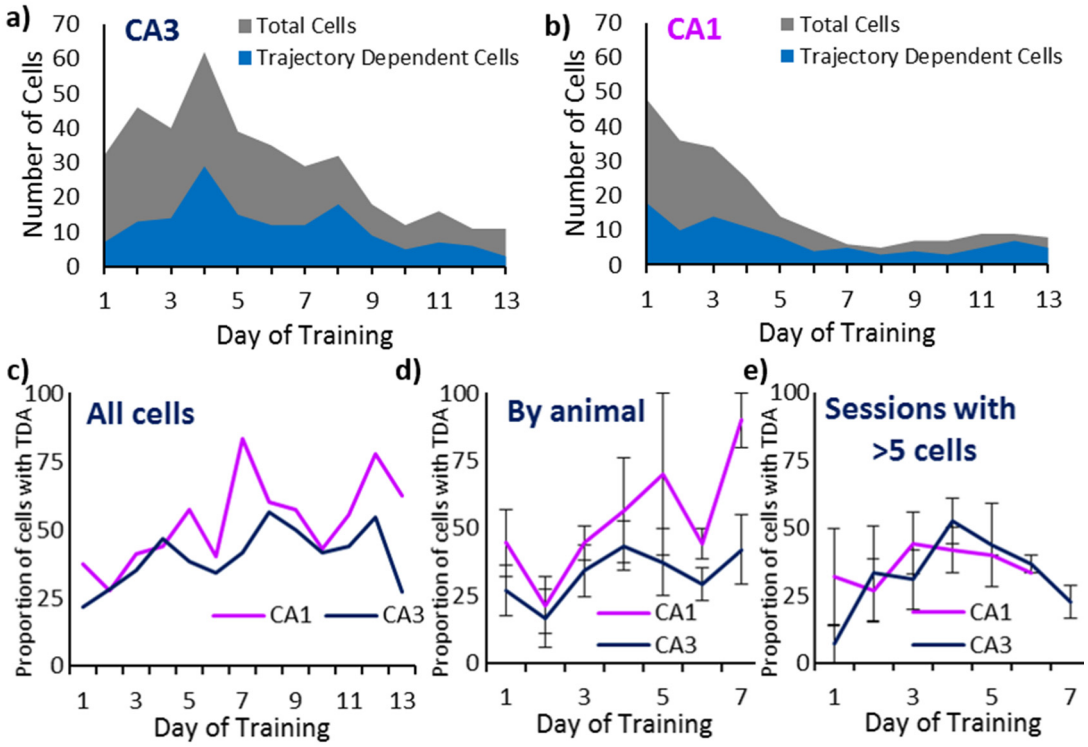


Figure 11: Trajectory dependent activity over time. a&b) total number of active cells per day (grey) and number of cells showing significant trajectory dependent activity (blue) in CA3 (a) and CA1 (b). c) proportion of cells showing significant trajectory dependent activity each day for CA3 (blue) and CA1 (magenta) d) mean and SEM proportion of cells per animal showing trajectory dependent activity averaged across all animals with active cells e) mean and SEM proportion of cells showing trajectory dependent activity per animal from sessions with more than five active cells.

To reduce bias caused by these data points, we repeated this analysis using only sessions in which 5 or more cells had been recorded (Figure 11e). There was no significant effect of either brain region [$F_{(1,25)}<0.1$; $p=0.79$] or day [$F_{(6,25)}=0.9$; $p=0.52$]. This suggests that the effect of brain region seen previously was driven by the highly variable data produced in sessions in which very few total cells were recorded, particularly as the overall trend of the graphs is more similar to the trend seen in the proportion of total cells per day.

In addition, the proportion of cells showing trajectory dependent activity for each session was plotted against the performance on that day (Figure 12a). The Pearson's correlation using all sessions with more than five cells was significant for CA3 cells

[r(32)=0.39; p=0.03] but not for CA1 cells [r(19)=0.21; p=0.38]. The highest correlation between performance and proportion of cells showing trajectory dependent activity was during the first four days which equates to the learning phase of the task. Figure 11g shows the data for the first four days only for CA3, the Pearson's correlation is highly significant [r(15)=0.70; p=0.004]. Conversely, if the data for the remaining days of training is correlated it is not significant [r(17)= -0.14; p=0.60]. For cells from CA1 neither the correlations for the learning phase (Figure 12b), nor the plateau phase (data not shown) are significantly correlated [learning: r(12)=0.29; p=0.36, plateau: r(7)= -0.54; p=0.21].

The data for development of trajectory dependent activity is not conclusive, but suggests a weak trend towards increased trajectory dependent activity on days when animals perform well at the win-stay task during initial learning of the task. There is no reliable evidence of a difference in developmental time-point of trajectory dependent activity between CA3 and CA1.

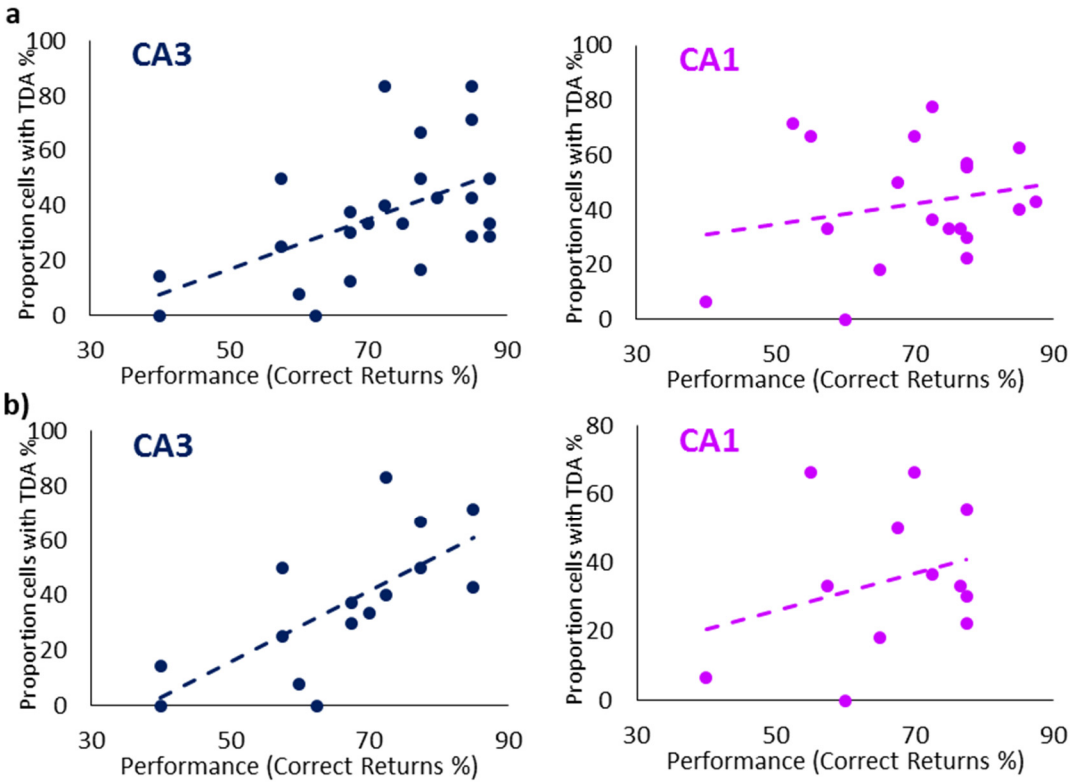


Figure 12: a) proportion of cells showing significant trajectory dependent activity for each session in which five or more active cells were recorded, plotted against the animal's performance on that session for CA3 cells (blue) and CA1 cells (magenta) b) proportion of cells showing significant trajectory dependent activity for each session during the first four days of training in which five or more active cells were recorded, plotted against the animal's performance on that session.

2.3.5 Histology

The locations of the electrodes were verified and all electrodes were within the regions intended. CA1 electrodes were very consistently positioned in the centre of CA1 (Figure 13). CA3 electrodes were much more variable, probably because of the additional depth within the brain, but all electrodes were located within CA3 (Figure 13). They were spread throughout CA3 with three electrodes within CA3c (the portion of the CA3 cell layer within dentate gyrus) and the remaining 5 electrodes located in CA3b (the middle region of CA3). No electrodes were located in CA2. Since trajectory dependent activity was found on all electrodes, and there was no consistent difference between electrodes, there is no indication of any difference along CA3. In the four animals with both CA3 and CA1 electrodes, two animals showed electrode tracks within approximately 120 μm of each other in the AP plane and the electrode tracks in the other two animals were no more than 300 μm apart indicating that they were not greatly different in their position along the septo-temporal axis of the hippocampus.

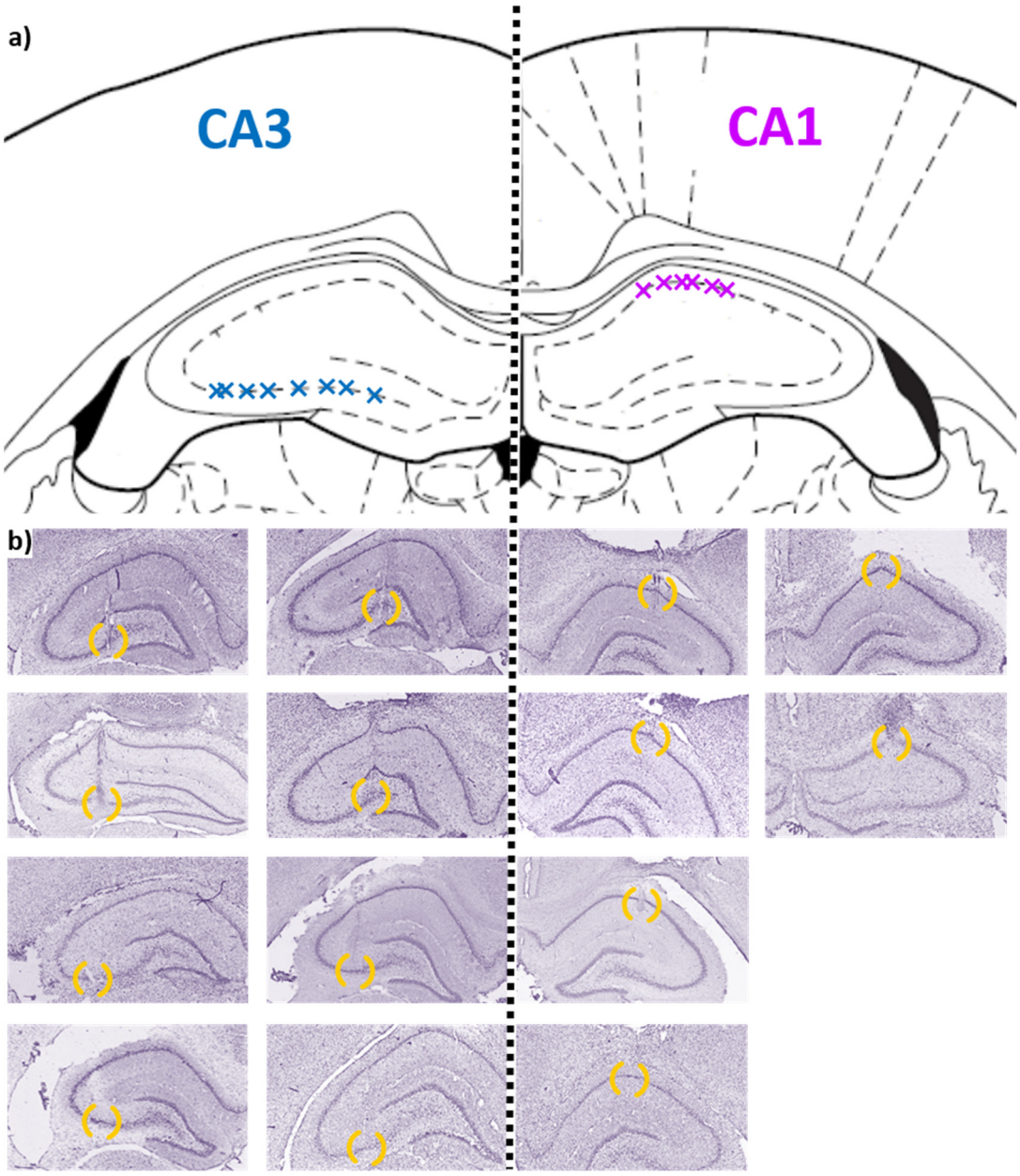


Figure 13: Histology: a) the approximate locations of the 8 CA3 electrodes (blue) and the 6 CA1 electrodes b) cresyl stained sections from each animal (the middle two columns show the sections from the four animals with electrodes in both CA3 (left) and CA1 (right)

2.4 Discussion

2.4.1 Trajectory dependent activity is present in CA3

Consistent with previous data, place cell activity in CA1 was modulated by trajectory on the double-Y-maze serial reversal task. The proportion of cells showing significant changes in firing rate across the different trajectory types was slightly higher than in the previous study. Ainge *et al*. found that 44% of CA1 place cells active in the maze showed trajectory dependent activity whereas 65% of CA1 place cells active in the maze showed significant trajectory dependent activity in one of the areas in our experiment. The novel finding in this experiment is that CA3 place cells was also modulated by trajectory. 43% of CA3 place cells active in the analysed areas of the maze showed significant trajectory dependent activity. This possibly implies that there is a slightly greater proportion of cells showing trajectory dependent activity in CA1 than in CA3. However this difference is driven by increased proportions of trajectory dependent activity in the side stems of the maze in CA1. In the central stem and goal boxes the proportion of cells from CA1 and CA3 which show significantly trajectory-modulated activity do not differ significantly. In addition, an alternative measure of trajectory dependent activity which compared the maximum change in mean firing rate between trajectory types showed no difference between CA3 and CA1 place cells for any of the maze areas.

In addition, the development of trajectory dependent activity as the task was learned was also analysed. Previous data shows that CA1 trajectory dependent activity is only present on this maze as the serial reversal task is learned, and develops around the timepoint at which learning occurs (Huang 2010, Stevenson 2011). This data does not show as clear an increase in trajectory dependent activity at the timepoint of learning the task, but there is still a strong correlation between the performance during the first days of training and the amount of trajectory dependent activity seen in CA3, which suggests a similar association between learning and trajectory dependent activity in CA3.

This result that trajectory dependent activity is present to a similar extent in CA3 and develops at a similar timepoint counters the idea that trajectory dependent activity

might originate in CA1, and suggests that some trajectory dependent activity originates earlier in the hippocampal circuit.

2.4.2 Comparison with previous studies

In this experiment place cells were categorised as either trajectory-dependent or trajectory-independent cells by performing an ANOVA or t-test on the firing rates in different trajectory types. The result in this experiment is that between 19% and 55% of CA1 place cells active in a maze area show trajectory dependent activity, while between 17% and 38% of CA3 place cells show trajectory dependent activity depending on the maze area analysed. There was no difference in the proportions of cells showing trajectory dependent activity in the central areas of the maze, and a small decrease in CA3 for side areas of the maze. This data is consistent with previous work by Bahar & Shapiro (2012) who also found evidence of similar levels of trajectory dependent activity in CA3 and CA1 place cells during a place learning task in a plus maze. 50% of active CA1 place cells and 52% of active CA3 place cells showed significant rate differences between trajectory types on the plus maze place learning task with serial reversals. However it contradicts data from Ito *et al*. (2015) who showed much less trajectory dependent activity in CA3 than in CA1 as animals performed a spatial alternation task with 55% of CA1 cells but only 18% of CA3 cells showing trajectory-dependent activity. Another measure of the extent of the trajectory coding is the maximum rate change between the mean firing rate for one trajectory type and the mean firing rate for the other trajectory type. In this measure also Ito *et al.* found much lower amounts of trajectory modulation with a mean of 33% rate change for CA1 but only 20% for CA3 and significantly different distributions of rate change. In contrast in the data presented here, there was no difference in the distributions of peak rate change with medians of between 45% and 58% rate change for CA1 cells and medians of 37% and 55% in CA3 cells. A direct comparison of the amounts of trajectory-dependent activity present in these three experiments is not particularly informative since previous research has shown high levels of variability in the proportion of trajectory-dependent activity in different tasks and even within a task. However since CA3 and CA1 place cells were directly compared in each of these

three experiments, the presence or absence of a difference between CA3 and CA1 in each experiment is meaningful. Both of the measures used to quantify trajectory-dependent activity in this experiment suggest little to no decrease in trajectory dependent activity in CA3 place cells compared to CA1 place cells, matching the data found by Bahar and Shapiro (2012) but inconsistent with the data found by Ito *et al.* (2015).

Ito *et al.* also found that lesioning or acutely silencing the nucleus reuniens, resulted in decreased CA1 trajectory dependent activity down to the levels seen in CA3. They therefore suggested that trajectory information entered the hippocampus via the nucleus reuniens, which only projects to CA1 but not CA3. Interestingly, Bahar & Shapiro used a hippocampus-dependent place learning task, while Ito *et al*. used a hippocampus-independent continuous spatial alternation task. The serial reversal double-Y-maze task used here is hippocampus-dependent (Ainge *et al* 2007a, Stevenson 2011). Collectively these data suggest that trajectory dependent activity may develop in different pathways depending on the specific task demands; particularly whether the task is dependent upon the hippocampus. In tasks which are not hippocampus-dependent, trajectory coding may occur in other brain areas such as the nucleus reuniens, prefrontal cortex or possibly the striatum. It is not clear whether trajectory-dependent activity in this other stream would also occur during hippocampus-dependent tasks and further recording from the nucleus reuniens is needed to determine this. In hippocampus-independent tasks the hippocampus may not be involved in generating trajectory information but may still combine trajectory information with spatial information at the level of CA1 in order to allow episodic memory or to deal flexibly with changes in task demands. Conversely, tasks which are known to require the hippocampus, may involve the hippocampus generating trajectory dependent activity and this may be the reason that the tasks require an intact hippocampus.

One other difference between the task used by Ito *et al*. and the task used here, is how the different trial types are arranged. In the continuous alternation task, rats are rewarded for alternating their trajectory with the result that the firing rate differences

are being compared between trial types which are nearly perfectly interleaved (Ito *et al*. 2015). In contrast in this study most of the trials of each type, particularly after the first few days of training, occur in blocks such that for example all the trials to goal 2 occur consecutively, followed by all the trials to goal 4 and so on. This could potentially predispose the data towards finding "trajectory dependent activity" since it is not possible to differentiate between time-dependent activity and trajectory dependent activity when trials are run in blocks. However, the trial types in the plus maze place task recorded by Bahar & Shapiro (2012) were not run in blocks but were pseudorandomly interleaved with no more than three trials in a row beginning in the same location. Since they also observed a high proportion of trajectory dependent activity in CA3, it seems unlikely that the arrangement of trials is the main reason for the difference in amount of trajectory dependent activity seen in this study and that of Ito *et al*.

2.4.3 Improvements and suggestions for future work

There are several ways in which this experiment could be improved. Firstly all the recordings from CA3 were taken from the right hemisphere, and all the CA1 recordings from the left hemisphere. The only reason for doing this was because the surgical set up made it easier to perform a clean implant on the right side of the brain. There was a clearer view of the brain surface on the right side of the brain which allowed the dura to be precisely removed and the brain surface to be cleaned before the electrode was inserted into the brain. Blood clots or an imperfectly removed dura can lead to poor signal quality, potentially preventing single-unit recording from the electrode. The priority was to record from CA3 since the presence of trajectory dependent activity has been well established in CA1 pyramidal neurons, and so all CA3 electrodes were implanted on the side of the brain which would be most likely to produce good results. In fact in our experiment many more cells were recorded from the CA3 electrodes than were recorded from the CA1 electrodes, which may have resulted from poor implantations on the CA1 side.

However, it has been shown that there are differences between CA3 projections to CA1 from the two hemispheres. Shinohara *et al*. (2008) reported that synapses between

CA3 projections and CA1 pyramidal neurons had different proportions of the different glutamate receptor subunits depending on which side of CA3 the projection originated. Postsynaptic spines receiving input from right CA3 projections showed a larger size, higher GluA1 density and lower GluN2B density. Evidence from Kohl *et al*. (2011) suggested that this difference in glutamate receptor composition produced a difference in plasticity. The authors showed that synapses between projections from the right CA3 to either side of CA1 showed less plasticity in response to optogenetic stimulus than synapses between projections from left CA3 to either side of CA1, and this difference was due to the greater prevalence of the GluN2B subunit in the synapses with left CA3 projections. This had an impact on mouse behaviour in that, while silencing either side of CA3 produced deficits in short term memory on a hippocampus-dependent memory task, only silencing of the left CA3 produced a long-term memory deficit in a hippocampus-dependent memory task with right CA3 silencing producing no impairment (Shipton *et al*. 2014). In the long-term memory task animals had to remember a rewarded location which was kept constant over several days, whereas the short-term memory task was a spontaneous alternation task in which animals only had to remember the previous location visited for a few seconds while they were picked up and placed back at the start location. The implication is, that no difference was seen between the inactivated hemispheres on the short-term task because no plasticity was required for the maintenance of the memory over such a short time period.

Since all of our CA3 recordings were performed in the right hemisphere, the trajectory dependent activity seen in CA3 pyramidal neurons may not be capable of inducing plasticity in CA1 although it should still be capable of inducing similar activity patterns in CA1 place cells. There is no reason to expect that less trajectory dependent activity would be seen in the left CA3 than was observed here in the right since there are no known differences in inputs or circuitry but this was not tested here. Long-term place field stability is known to be dependent upon plasticity, as it can be blocked by NMDA receptor antagonists (Kentros *et al* 1998). However NMDA receptor blockade does not affect already established place field firing, nor place field remapping upon

entering a new environment. It is not known whether trajectory dependent activity is dependent upon plasticity. The finding by Ito *et al*. (2015) that acute inactivation of the nucleus reuniens immediately reduces trajectory dependent activity in CA1 would suggest that trajectory dependent activity (at least in CA1 pyramidal cells in a continuous alternation task) is not a learned firing pattern but rather reflects the ongoing activity of the inputs. Therefore the result described here in CA3 in the right hemisphere (which is thought to induce activity but not plasticity in CA1) may not be surprising. The question of whether trajectory-dependent component of place field activity shows long-term stability has never been addressed. There is some evidence from Bahar and Shapiro (2012) that stability of trajectory dependent activity can occur across days; 50% of cells showing trajectory dependent activity in one session would show trajectory dependent activity across two consecutive days of recording, with the other 50% of cells showing a change in trajectory dependence between days. Place field stability between sessions was much higher with between 69-86% of cells showing stable fields between sessions (Bahar *et al* 2011). However in their experiment, a changed session (in which either the goal locations or the room cues were changed) occurred in between the two standard sessions so it is not clear whether the large amount instability of trajectory-dependent activity is due to a lack of stability in the journey coding or whether it is the result of interference from the other task. It would be interesting to record the same place cells across multiple days in the double-Y maze task described here. Since place field stability across days requires plasticity, if the same trajectory dependent fields were seen across multiple days it might suggest that this aspect of place field firing can also be stored long-term by plasticity within the place cell circuit. However unfortunately in this study there was not enough evidence of electrode stability across consecutive sessions to allow the identification of place cells across days. Alternatively, this idea could be tested by blocking plasticity, however in a hippocampus-dependent task this would probably impair behaviour confounding the results.

The conflicting results from this experiment, Bahar and Shapiro (2012) and Ito *et al*. (2015) suggest an interesting link between hippocampal-dependence of tasks and the

presence of trajectory dependent activity in CA3. The present and past results on the extent of trajectory-dependent activity in CA3 can be explained by the hypothesis that in hippocampus-dependent tasks CA3 place cells develop trajectory-dependent activity which is then passed on to CA1 place cells, while in hippocampus independent tasks, CA3 does not develop high levels of trajectory-dependent activity. Instead, trajectory-dependent activity in CA1 in these tasks comes from the nucleus reuniens and so removing this input leaves only the small levels of trajectory-dependent activity seen in CA3 even in hippocampus-independent tasks. It would be interesting to follow this up by looking for CA3 trajectory dependent activity in other hippocampus-independent tasks, for example the response task on the plus maze. In addition, a better understanding of the content of the reuniens input to the hippocampus might be gained by working out what sort of tasks require the presence of this input. This will be explored further in Chapter 3.

In conclusion, this chapter provides more evidence that CA3 place cell activity is modulated by an animal's trajectory. These results confirm previous findings of trajectory dependent activity in CA3 place cells (Bahar and Shapiro 2012). The conflicting result from Ito *et al* (2015) may be explained by the hippocampal-independence of the task used in that study, and with the results described here suggest that the presence of trajectory dependent activity within CA3 may coincide with the hippocampus-dependence of the task. If this correlation proves to be consistent in other tasks, it could indicate that the conjunction of spatial and trajectory information in CA3 place cell activity is the mechanism by which hippocampus-dependent tasks are learned or performed. The reason such tasks are dependent upon an intact hippocampus might not only be because the tasks require the representation of an animal's current location, but because information about current location must be combined with encoding of the spatial trajectory needed to reach the goal.

3 The role of the nucleus reuniens in hippocampus-dependent tasks

3.1 Introduction

The nucleus reuniens is a midline thalamic nucleus, and is a major source of thalamic input to the hippocampus. Because of its reciprocal connections with both the prefrontal cortex (McKenna *et al*. 2004, Vertez *et al.*2006) and hippocampus (Wouterlood *et al*. 1990, Dolleman-Van der Weel and Witter 2000), it has been suggested as a relay between the prefrontal cortex and hippocampus, regulating the flow of information between them. This connection is important because there is no direct projection from the prefrontal cortex to the hippocampus.

Later work showed that this potential relay is a one-synapse connection; neurons from the prefrontal cortex synapse directly onto excitatory neurons in the nucleus reuniens which project onto CA1 (Vertez *et al.* 2007). Earlier electrophysiological work had shown that stimulating the nucleus reuniens produced a greater effect on CA1 than stimulating CA3 (Bertram and Zhang. 1999), suggesting that this is a strong excitatory projection, although other work suggested that the projection could not directly produce spikes from CA1 pyramidal neurons but rather acted by subthreshold depolarization of the pyramidal neurons and excitation of interneurons (Dolleman-Van der Weel *et al*. 1997).

Since this projection is potentially the most effective route of information flow from the prefrontal cortex to the hippocampus, it would be interesting to establish what information from prefrontal cortex it is contributing to CA1 pyramidal neurons. Recently there have been two exciting new discoveries about the information encoded by nucleus reuniens neurons. Firstly Jankowski *et al*. (2014) found head-direction cells within the nucleus reuniens. Secondly, and more directly relevant to our question, Ito *et al*. (2015) found that neurons in both the nucleus reuniens and prefrontal cortex show trajectory dependent activity. Unlike the trajectory dependent activity in hippocampal place cells, this activity is not spatial but rather encodes different trajectories as a whole. Removing the nucleus reuniens input to CA1 reduces the

trajectory dependent activity seen in CA1 place cells, which strongly suggests that the role of the nucleus reuniens during this task is to provide information about trajectory to place cells, where it can be integrated with information about the animal's current location. This information may allow the animal to make predictions about the results of their actions. Alternatively, it may be a feedback mechanism allowing the hippocampus to integrate the decisions with location, enabling disambiguation of memories formed in the different trajectories. Trajectory dependent activity has been observed in several spatial tasks, both hippocampus-dependent and independent. The task used in the paper by Ito *et al.*(2015) was not hippocampus-dependent (Ainge *et al.*2007b). This prevented any link being made between reductions in trajectory dependent activity and learning or performance of the task. As trajectory dependent activity may represent the memory of how to solve a trajectory based task, exploring the effects of nucleus reuniens lesions on a task in which trajectory dependent activity is seen but which is also hippocampus-dependent, might throw light on the roles of both the nucleus reuniens and trajectory dependent activity itself. The first experiment described here aims to do this, by testing the effects of nucleus reuniens lesions on the acquisition and performance of the double-Y-maze serial-reversal task which is known to be both hippocampus-dependent and induce trajectory dependent activity (Ainge *et al*. 2007a, Stevenson 2011). It is hypothesised that if the nucleus reuniens is providing the trajectory information necessary for the generation of trajectory dependent activity in CA1 place cells, and if trajectory dependent activity is necessary for learning trajectory-based hippocampus-dependent memory tasks, then removing the nucleus reuniens will cause a deficit in either learning or performing the double-Y-maze serial-reversal task.

Since the role of the hippocampus in navigation is well documented, exploring the effect of nucleus reuniens lesions or inactivation on navigational tasks which are known to require an intact hippocampus might provide other clues as to what sort of information or regulation the nucleus reuniens might be providing. The role of the nucleus reuniens in spatial working memory has been tested in several different tasks and mazes. Reuniens lesions impair learning a win-shift radial maze task and a delayed

non-matching to position task in an operant chamber but do not impair a variable choice radial maze delayed non-matching task (Hembrook and Mair 2011, Hembrook *et al.*2012). The first two tasks require both the hippocampus and prefrontal cortex and were impaired by nucleus reuniens lesions, while the last task does not require both the hippocampus and prefrontal cortex and was not impaired by nucleus reuniens lesions. It has therefore been suggested that nucleus reuniens lesions may be involved in spatial working memory tasks only when these involve both the prefrontal cortex and hippocampus, but not when the task is only dependent on the hippocampus (Hembrook *et al.*2012). Since delayed alternation on the T-maze is both hippocampus-dependent (Dudchenko *et al*. 2000) and pre-frontal cortex-dependent (Shaw and Aggleton 1993), the aim of the second experiment was to test the contribution of the nucleus reuniens to spatial working memory using a forced-choice delayed-alternation task with a variable delay on a Y-maze.

Both the serial reversal double-Y-maze task and the forced choice delayed alternation T-maze task could be solved either egocentrically or allocentrically. Reference memory for a hidden platform in the water maze is usually used to test allocentric navigation because taxon navigation is prevented by reducing the availability of local olfactory, tactile or visual cues which could be used as beacons, and egocentric navigation is prevented because the start location changes on every trial (Morris *et al.* 1982). Previous data on the role of the nucleus reuniens in solving watermaze tasks have been somewhat contradictory. Nucleus reuniens lesions or inactivation do not impair reference memory in the water maze according to Dolleman-van der Weel *et al*. (2009), Loureiro *et al*. (2012) and Cholvin *et al*. (2013). However in another study, reuniens inactivation did impair both reference memory and a delay-match-to-place task in the water maze irrespective of whether the inactivation occurred during or after acquisition or before retrieval (Davoodi *et al*. 2009). As this experiment lacked anatomical controls, it is possible that the inactivation was not restricted to the nucleus reuniens and that the effects seen were due to more widespread inactivation within the thalamus. In addition, Loureiro *et al*. found that although nucleus reuniens lesioned rats were initially unimpaired, they had forgotten the platform location after 25 days,

suggesting a role for the nucleus reuniens in consolidation but not acquisition or performance of the reference memory task. The third experiment carried out here seeks to answer the question of whether the nucleus reuniens is required for allocentric navigation, since both the double-Y-maze and delayed alternation tasks could be solved by either an allocentric or an egocentric strategy. If the reuniens is involved only in allocentric tasks but not egocentric tasks, this should result in a deficit in the water maze reference memory task even if the double-Y-maze task is unimpaired.

Another potential role for the nucleus reuniens is in strategy selection. In order for spatial strategy selection to occur, it has been hypothesised that the prefrontal cortex may need to communicate with the hippocampus in order to either select a specific strategy or to judge whether a strategy is working. There are two main strategies that can be used in maze tasks. An allocentric or 'place' strategy involves navigating to one specific location, no matter what the start location; for example always going to the east arm on a plus-maze. An egocentric or 'response' strategy involves navigation based on remembering one or more body turns; for example, always turning right on a plus maze. Lesions to the nucleus reuniens produced deficits in shifting to using a place strategy in a double-H maze (Cholvin *et al*. 2013). However the deficit in strategy switching may depend on the type of strategy being selected; data from the water maze showed that nucleus reuniens lesioned animals were quick to shift to a random searching strategy during probe trials rather than remaining above the platform location (Dolleman-van der Weel *et al*. (2009), suggesting that in this task, nucleus reuniens lesions increased behavioural flexibility in response to changes in task conditions. Conversely, Prasad *et al.*(2013) suggested that the reuniens might have more of a role in inhibition rather than flexibility, as reuniens lesions reduced the ability to inhibit premature responses in a 5 choice reaction time task. The prelimbic and infralimbic cortices which project to the nucleus reuniens (Varela *et al*. 2014) are thought to control selection between different strategies (Rich and Shapiro 2007, 2009). Rich and Shapiro trained animals on either a place strategy or a response strategy on the plus maze. They found that inactivation of the prelimbic and infralimbic cortices caused deficits remembering the current strategy 24 hours after switching

between familiar strategies, although after extensive training these deficits disappeared because performance became independent of the previous day's training. In addition, they found that the activity in the prelimbic and infralimbic cortex coded for the type of strategy being used. Prelimbic activity anticipated the strategy switch suggesting that it may be involved in selecting or promoting new strategies, while infralimbic activity may help to stabilise strategies. Since the hippocampus is needed for learning the plus-maze place task, it could be hypothesized that the reuniens would be necessary for switching to a place strategy since this should require communication between the prelimbic cortex and hippocampus. The final experiment tests whether nucleus reuniens lesions have the same effect as prefrontal cortex lesions on switching between a caudate nucleus-dependent response strategy and a hippocampus-dependent place strategy (Packard and McGaugh 1996), using the same plus maze tasks used previously by Rich and Shapiro (2007).

3.2 Methods

3.2.1 Subjects

12 male Lister Hooded rats (Charles River Laboratories, UK) were used in all experiments. They weighed between 260 and 300g at the time of surgery. Rats were group-housed in cages of four and maintained on a 12 hour light/dark cycle. Rats were pseudorandomly assigned to experimental groups such that each cage contained at least one sham and two lesion animals, and all behavioural testing was performed with experimenters blind to the experimental group of the animals. All surgeries, training and testing were performed during the light phase of the cycle. After approximately one week's recovery from surgery all animals were food restricted to 85% of their free-feeding weight for the remainder of the experiment. All procedures complied with the Animals (Scientific Procedures) Act, 1986, and were approved by the Named Veterinary Surgeon.

3.2.2 Surgery

Animals were anaesthetized with isofluorane and placed in a stereotaxic frame, and anaesthesia was maintained with isofluorane and oxygen administered via a nose cone. Small Animal Rimadyl was administered subcutaneously for analgesia at a dose of 0.08ml/kg-body-weight. A midline scalp incision was made to expose the skull between bregma and lambda . Holes were drilled on the right side of the brain to expose dura. For the control animals (n = 5), 3 holes were pierced in dura using a sterile needle. Dura was then covered with sterile gel foam and the skin was sutured. For the lesion animals (n = 7), 3 injections of ibotenic acid (10mg/ml) were made along the anterior-posterior axis of the nucleus reuniens using a Hamilton syringe at 10°from vertical in the M-L plane pointing in towards the midline (See Table 1). Dura was then covered with sterile gel foam and the skin was sutured. The animal was placed on a heat bench at 30°C until it regained consciousness and then for a further hour of recovery.

AP (mm)	ML (mm) (at 10°)	DV (mm) (at 10°)	Volume
1.8	1.18	6.90	0.05 µl
2.5	1.23	7.21	0.05 µl
3.2	1.23	7.21	0.05 µl

Table 1. Nucleus reuniens lesion coordinates

3.2.3 Apparatus

Double Y maze

The maze was built out of wood and painted with black paint. It consisted of a start box, 3 choice boxes and 4 goal boxes connected by alleyways (Figure 1a). All the boxes were octagonal and measured 25 cm across with 30 cm high walls. The alleyways were 25 cm long and 8 cm wide with 10 cm high walls. Barriers 30 cm high were used to prevent animals from leaving the start box and goal boxes between trials. Each goal box contained a food well which either contained Coco Pops (Kellogg's) or was empty. Beneath a metal grating were additional inaccessible coco-pops to ensure that all food wells smelled the same. A camera located directly above the maze and connected to a DVD recorder was used to record all trials.

Single Y maze

The same maze components were used as in the double Y maze task but arranged in a single Y maze configuration with one start box, one choice box and two goal boxes

(Figure 1b). Again a camera located directly above the maze and connected to a DVD recorder was used to record all trials.

Water maze

The water maze was a circular pool of water 2m in diameter (Figure 1c). The pool water was maintained at a temperature of 24+/-1°C and was mixed with 400ml floor screed latex to make it opaque. A circular Atlantis Platform (Spooner *et al.* 1994) 12 cm in diameter was positioned in one of two locations 2 cm below the surface of the water. During Probe trials this platform was positioned on the bottom of the pool and was programmed to rise to 2 cm below the surface of the water after 60 seconds.

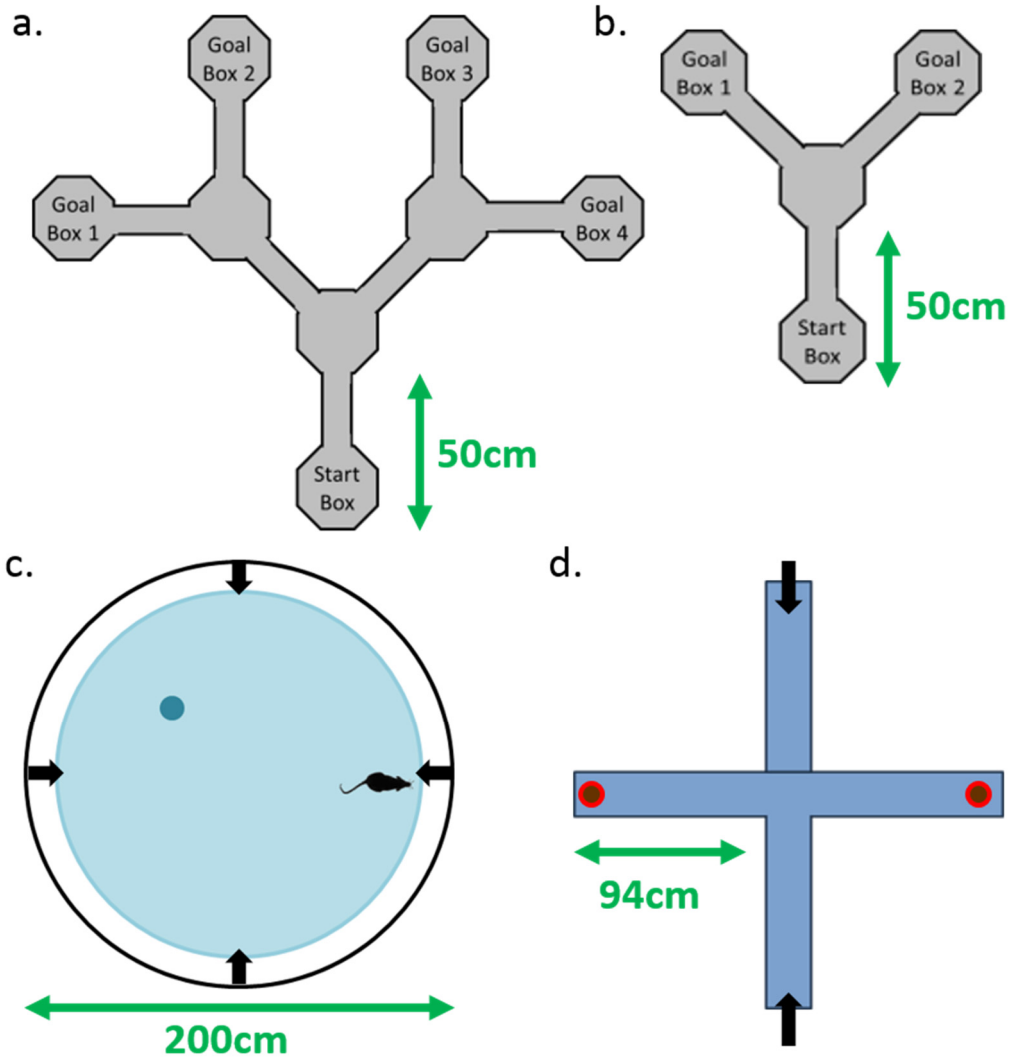


Figure 1: Dimensions and plan of the apparatus: a) double-Y-maze b) Y-maze c) watermaze d) plus maze

A camera connected to a DVD recorder and to data acquisition software (Watermaze©) was positioned directly above the pool and was used to track the rats' position.

The water maze was located in a cue-rich environment with several large cues suspended from the walls and ceiling and one entire wall was a contrasting colour due to black window blinds.

Plus maze

A plus maze was constructed using wood for the floors of the maze and Plexiglas for the walls. The alleys were 94 cm long and 9 cm wide with 14 cm high walls (Figure 1d). A Plexiglas barrier was placed in entrance to the alley opposite to the start alley to prevent animals entering it. A food well was placed in each of the east and west arms. On a given trial only one of these contained a food reward of 3-5 coco pops (Kellogg's). Both food wells also contained inaccessible coco pops (Kellogg's) under a grill to prevent rats from using odour to solve the task. A camera located directly above the maze and connected to a DVD recorder was used to record all trials.

3.2.4 Behavioural Experiments

Timeline of experiments

Behavioural experiments commenced 9-14 days following surgery. During this recovery period, animals were handled and habituated to being brought to the testing room. After the first week of recovery, during which time all animals had regained their presurgery weight, they were placed on food deprivation. Animals received three weeks of training on the double Y maze task followed by two weeks of training on the delayed alternation task. They were then trained for two weeks on the water maze task and then four weeks of training on the strategy-switching task in the plus maze (Figure 2a). Animals were killed the week after the last experiment was completed with the exception of two of the lesion animals who started having seizures and so were euthanized before completion of the plus maze task. Some handling and most of the training on the double Y maze, delayed alternation task and water maze were performed by Thomas Ripard and Georgy Yukhnovich.

Double Y maze serial-reversal task

Animals were run on the maze for 15 days. Each day animals received up to four blocks of trials. On each trial only one food well contained a food reward. The same goal location was rewarded within each block of trials (a win-stay rule) but a different goal location was rewarded on each block (a lose-shift rule). Each block continued for 9 trials after the first trial that the animal entered the rewarded goal box (Figure 2b).

In each trial, animals were placed in the start box of the maze while the maze was wiped down with warm soapy water. The barrier was then removed and animals were allowed to explore the maze in a forwards direction (no U-turns) until they entered one of the goal boxes. If this box was an unrewarded box, animals were held in the box for five seconds before being replaced in the start box. If the box was rewarded the animals were allowed to eat the reward for a few seconds before being returned to the start box.

For each trial, the experimenter recorded which goal box was entered and the latency to find it. In addition, vicarious-trial-and-error (VTE) was assessed on each trial both by the experimenter at the time and then validated by re-watching a video of the trial. VTE refers to a behaviour seen at choice points in a maze, where an animal hesitates and moves their head from side to side as if comparing the two options (Meunzinger, 1938). A VTE was counted each time the animal moved his head from facing one alleyway towards the other alleyway (Figure 2c).

Single Y-maze delayed alternation task

Rats were placed in the start box while one of the two goal boxes was randomly opened and the other blocked off. The rat was allowed to enter the open goal box and consume a food reward. There was then a variable delay period before the animal was allowed to make a choice between either of the two goal boxes (Figure 2d). The animal was rewarded for entering the other box. The delay was initially 15 seconds, then 60 seconds, then 5 minutes. Rats were given 10 trials per day with three days at each delay length. The intertrial interval was 5 minutes for all trials. The box chosen and the latency to enter the chosen box were recorded for each trial.

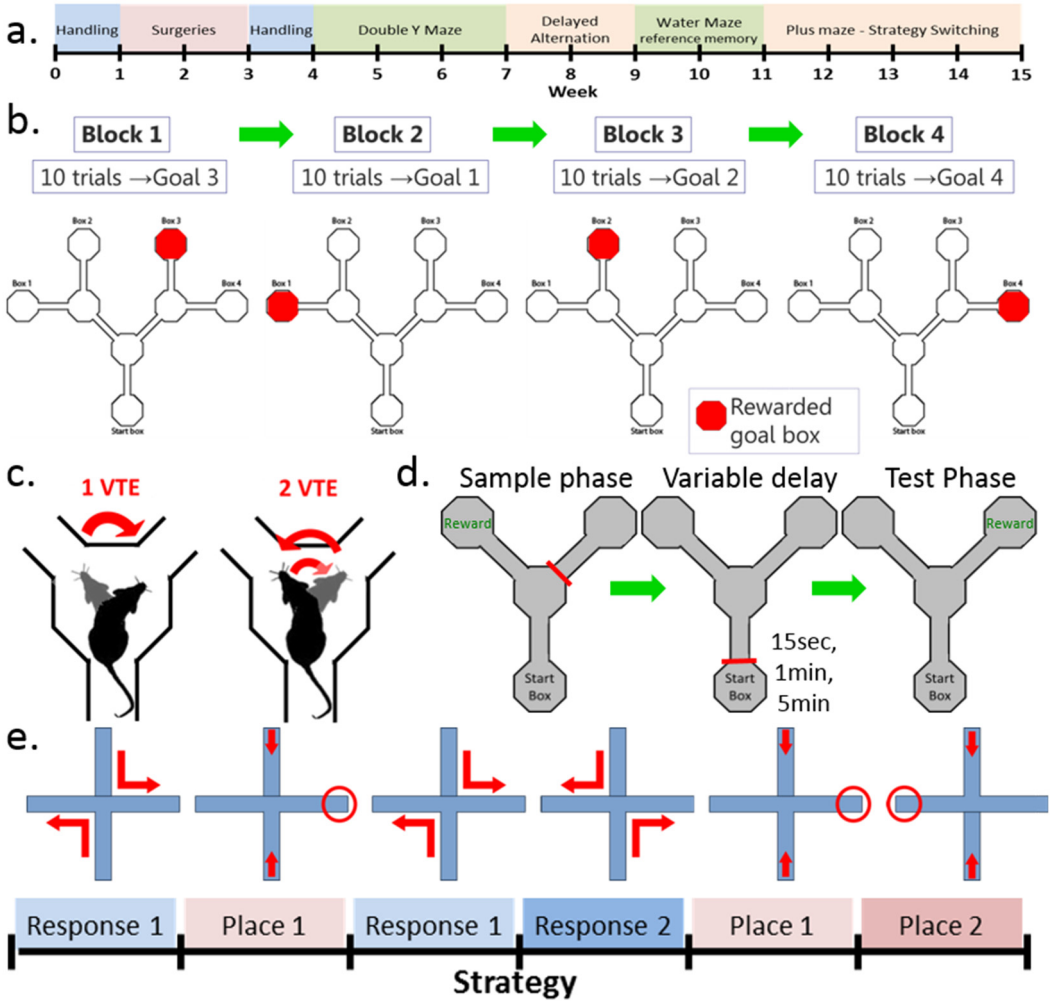


Figure 2: Behavioural Experiments a) timeline of experiments b) one day of training in the double-Y-maze serial-reversal task; each day of training consists of 4 blocks. On each block a different goal is rewarded such that each location is rewarded for one block per day. The block began when the reward was moved to a new location and consisted of a number of searching trials where the animal ran from the start box to one of the goals. Following the first trial on which an animal found the correct goal, the animal was given 10 more trials to that goal, after which a new block began c) how VTE was calculated; one VTE consisted of one head movement either left-right or right-left, 2 VTEs consisted of either left-right-left or right-left-right d) forced-choice delayed-alternation protocol: each trial consisted of one sample phase in which one goal was accessible. After consuming a reward in the sample goal, the animal was then placed in the start box for a variable delay. This was followed by the test phase in which the animal was given free choice between the two goal boxes e) example sequence of strategies in the plus maze (arrows indicate rewarded response, circles indicate rewarded place), specific responses and places were counterbalanced such that half the animals started with the opposite response strategy, or had the opposite location rewarded as place 1, the order of strategy types: response, place, response, response-reversal, place, place-reversal was not counterbalanced)

Water maze reference memory and reversal tasks

In initial reference memory training, rats were trained with 6 trials per day for 6 days with one platform location situated either in the northwest or southeast of the water maze. Half the animals in each experimental group were assigned to each platform location. On a given trial a rat was placed into the water maze facing the near wall in one of four pseudorandom start locations: north, east, south and west. Each day two sequences of 6 start locations were created which were opposites of each other; for example: N.S.W.N.E.W and S.N.E.S.W.E. Each start location was used either once or twice each day. The start locations were matched between groups, but half of the animals in each experimental group and platform location were assigned to each of the two sequences for the day. The experimenter then pressed a button to start the trial and quickly moved out of sight of the animal. The trial would continue until the animal reached the platform or until 120 seconds had passed. If the rat had not reached the platform in this time, the experimenter guided the animal to the platform by hand. The rat was then left on the platform for 20 seconds, before being picked up and placed in a recovery cage under an infra-red lamp for an intertrial interval of 10 minutes. Any faeces were removed from the water, and the water was stirred between trials to remove odour cues. On the first trial of days 2, 4 and 6, a probe trial was conducted in which the Atlantis platform remained on the bottom of the pool for the first 60 seconds of the trial, after which it rose to the usual depth so that the animal could be rewarded for finding it.

Following the 6 days of reference memory training, rats were given a break for one day, and then reversal training began. Reversal training was performed exactly as described above, except that the platform was located on the opposite side of the pool. Again, probe trials were conducted on days 2, 4 and 6.

During all water maze trials, the rat's position was tracked using an overhead camera connected to a DVD player and the Watermaze© tracking program. This program recorded the animal's position at a frequency of 10 Hz as well as calculating other parameters for each trial, such as latency and path length to the platform. For probe

trials, the percentage of time spent in each quadrant for the first 60 seconds was also calculated using this program.

Plus maze strategy-switching task

All rats were trained on the four possible strategies in this sequence; Response 1, Place 1 (strategy switch), Response 1 (strategy switch/repeat), Response 2 (response reversal), Place 1 (strategy switch/repeat), Place 2 (place reversal) (See Figure 2e). Responses 1 and 2 were counterbalanced such that for half of the rats in each experimental group the first response was turn left and for half it was turn right. Places 1 and 2 were also counterbalanced such that half of the animals in each group had east as the first place and half had west first. Rats were given 24 trials a day with 12 trials starting in the north arm and 12 trials starting in the south arm arranged in a pseudorandom order (No more than 3 consecutive trials from one start arm). On each trial the rat was placed in the designated start arm for that trial and the opposite start arm was blocked making the maze into a T maze. The rat was allowed to enter either of the two reward arms (East or West) but was prevented from going back with a barrier once they had completely entered one box. For the response strategy, rats would either be rewarded for turning right or for turning left (counterbalanced within each group). For the place strategy, rats would either be rewarded in the East arm or in the West arm. Once the rat reached the food well, it was either allowed to eat several coco-pops or blocked there for 5 seconds before being placed in the designated start arm for the next trial while the maze was cleaned. After every trial, the maze was wiped down with soapy water, and after every 5-6 trials the maze was rotated by 90° to prevent the use of local intramaze cues. At each stage, rats were given 24 trials per day until they reached a criterion performance of 10 correct consecutive trials. If the rat's last 7 trials of the day were correct he was given up to three more trials to attain criterion. After reaching criterion, the rat was started on the next strategy the following day. The experimenter recorded which goal arm the animal entered on each trial, as well as calculating the number of individual trials taken before the animal reached the criterion of 10 correct consecutive trials.

3.2.5 Histology

Following completion of data collection, rats were anaesthetized with isofluorane and given a lethal dose of sodium pentobarbitol (Euthatal, Meridal Animal Health, UK). The tissues were fixed by transcardial perfusion of 0.9% saline followed by ice-cold 4% paraformaldehyde. The brains were then extracted and stored overnight at 4°C in 4% paraformaldehyde before being cryoprotected in 30% sucrose solution. They were then stored in a -70° freezer. Brains were sectioned coronally at 32 μm thickness with a cryostat-microtome. Half of the sections were mounted on polysine slides (Thermo Scientific, UK), stained with 0.1% Cresyl Violet, and coverslipped in DPX (Sigma-Aldrich, UK). One in eight sections were reserved for immunohistochemistry. Sections were incubated in Mouse NeuN antibody (MAb377, Millipore, UK) diluted 1:1000 in T-PBS for 30 hours, rinsed, and then incubated in Donkey Anti-mouse IgG Cy3 (Millipore, UK) diluted 1:200 in T-PBS for 2 hours. DAPI was used as a counterstain. Sections were then mounted and photographed at 20x magnification using Image-Pro Plus (Media Cybernetics, USA).

Quantification of lesions was performed using both the nissl-stained sections and the NeuN stained sections. The lesion area for each animal was drawn onto an electronic copies of the relevant Paxinos and Watson rat brain atlas plates (1998) using CorelDRAW, and then an automated pixel count was performed using a custom script (Mitchell and Dalrymple-Alford, 2006) which calculated approximate lesion volumes based on the distance between each panel of the atlas. Because of distortion of the tissue around the lesion site, a fully automated process based on the micrographs themselves would have introduced other errors and so a visual assessment of the lesion size was necessary when defining the lesion area.

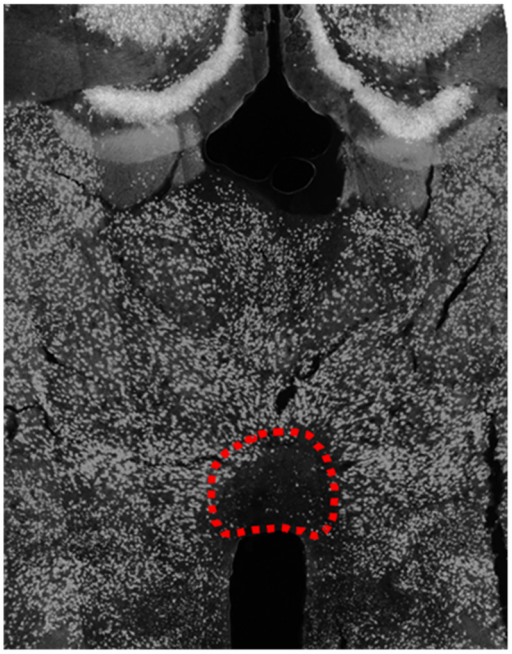
3.3 Results

3.3.1 Histology

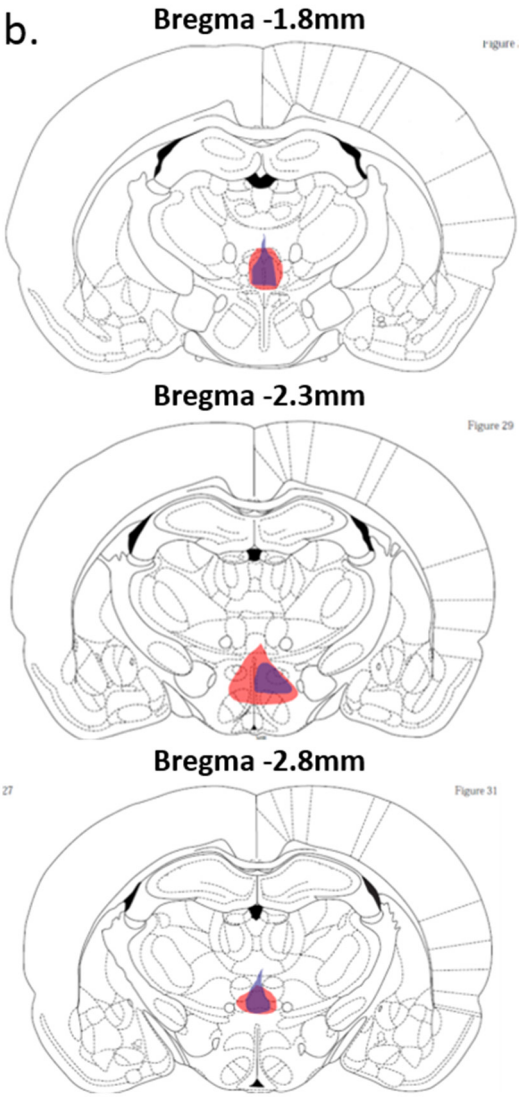
Histological quantification of lesions was performed upon sections stained with either Nissl staining or NeuN. Nissl stained sections allowed easy identification of the location of the lesion relative to adjacent thalamic areas, while NeuN allowed easy

identification of whether cells within the lesion area were spared neurons or glial cells (Figure 3a). Semi-automated quantification showed that the nucleus reuniens was selectively lesioned in 5 of the 7 animals. One of the other two rats had a lesion which missed the reuniens entirely; while in the other rat, the lesion included adjacent areas of the thalamus. Both animals were excluded from further analysis. The five animals included in the study had lesions ranging from 48% to 83% of the total reuniens volume. The extent of the largest and smallest lesions is shown in (Figure 3b) superimposed onto the relevant plates of the Paxinos and Watson Brain Atlas. Sparing to the nucleus reuniens was most pronounced at the extreme anterior of the nucleus

a.



b.



c.

	Anterior Reuniens 6 sections	Posterior Reuniens 6 sections	Total Reuniens
5 Lesion animals	73%	100%	**83%**
	79%	76%	**78%**
	18%	94%	**64%**
	30%	78%	**49%**
	18%	94%	**48%**

Figure 3: Histology a) example Neu-N section b) extent of largest (pink) and smallest (blue) lesions c) lesion extent at anterior and posterior of reuniens

with damage to the anterior half ranging from 18% to 79%. The posterior half of the nucleus reuniens showed a more consistent lesion ranging from 76% to 100% (Figure 3c). None of these lesions showed extensive damage to other areas. All results were examined for differences between the three animals with large lesions compared with the two animals with smaller lesions. Where no differences were found, all animals were included in the results.

3.3.2 Double-Y-maze serial-reversal task

Animals were trained on the serial-reversal task for 15 days. Three measures were used to assess performance during each day of training. Overall performance, measured the percentage of total trials on which the animal was rewarded. Percentage correct returns measured how consistently the animal returned to the rewarded box once it had been found once within a block of trials (averaged across all four blocks within a session),

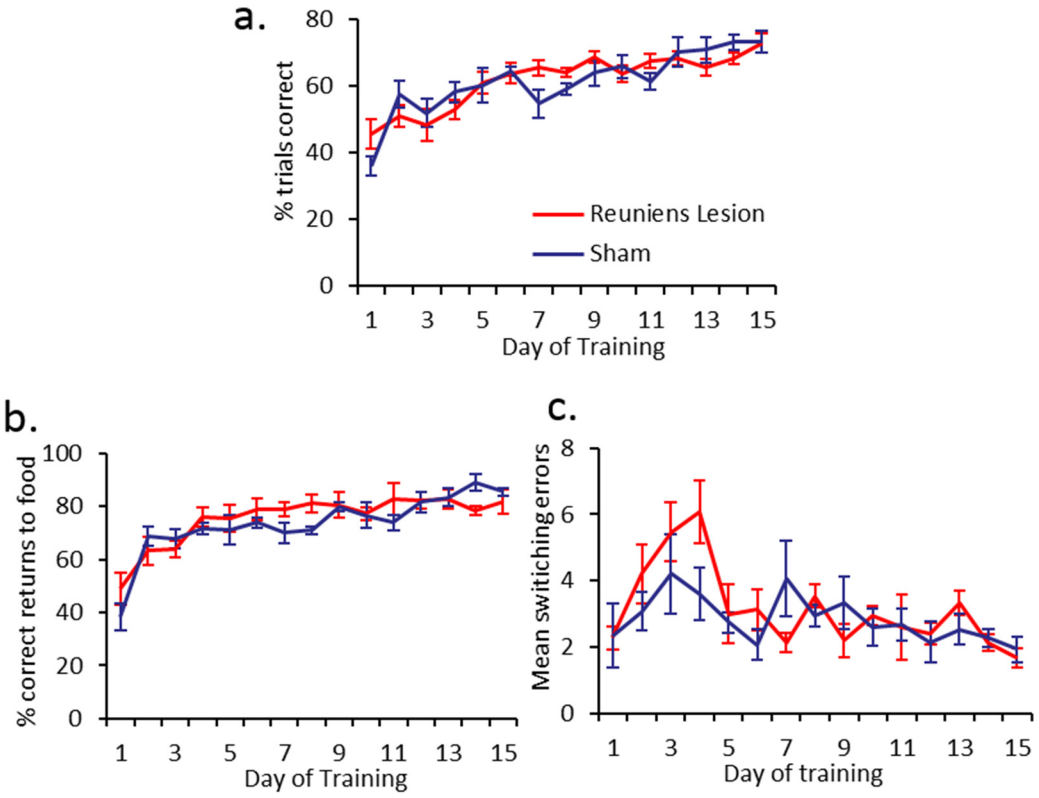


Figure 4: Double Y maze performance a) percent correct trials over whole session (+/- SEM) b) percent correct returns to food (+/- SEM) c) mean number of trials taken to switch to new box (+/- SEM)

and was therefore assessing the win-stay component of the task. Switching errors measured how many trials it took for an animal to find the new box after one unrewarded trial to the previous rewarded box when its location moved between blocks (averaged across all three switches within a session), and so was assessing the lose-shift component of the task. Animals in both groups showed improvement over time as shown by a strong effect of day [RM-ANOVA Day: $F_{(14,112)}$=16.4; p<0.001] on overall performance (Figure 4a). There was no significant difference between the groups [RM-ANOVA Group: $F_{(1,8)}$<0.1; p=0.82] and no interaction between the group and day of training [RM-ANOVA Group*Day: $F_{(14,112)}$=1.8; p=0.06]. In addition, no differences between groups were observed in percentage correct returns (Figure 4b) suggesting no deficit in the win-stay component of the task [RM-ANOVA Group $F_{(1,8)}$=0.1; p=0.35] nor in the rate of learning this aspect of the task [RM-ANOVA Group*Day $F_{(14,112)}$=1.5; p=0.14] although animals in both groups showed learning [RM-ANOVA Day: $F_{(14,112)}$=15.4; p<0.001]. There was no overall difference between the groups in the lose-shift aspect of the task [RM-ANOVA Group**:** $F_{(1,8)}$ = 0.9; p=0.37**,** Group*Day: $F_{(14,112)}$ = 1.2; p=0.26] although there was an effect of day showing that animals learned over time [RM-ANOVA Day: $F_{(14,112)}$ = 3.5; p <0.001]. There appeared to be slightly more switching errors on initial days of training (Figure 4c), which would correspond to the days of acquisition as animals learned the task before their performance reached asymptote, but this did not reach significance [RM-ANOVA First five days $F_{(1,8)}$=4.7; p=0.06].

Since the reuniens has been suggested to have a role in inhibition, we recorded and compared the prevalence of vicarious-trial-and-error (VTE), and the run-times in the two groups. VTE was most prevalent in both groups during the first few days (Figure 5a) after which it plateaued at around 1 VTE per 5 trials, or 0.2 VTEs/trial. As has been reported previously (Bett *et al*. 2012) there were more VTEs during searching trials (before the food had been found in a location) than during returning trials (during the ten trials once the animal had found the food location once) (data not shown [$F_{(1,8)}$=42.8; p<0.001]). The lesion group did not show significantly fewer VTEs than controls during searching trials (Figure 5b) [RM-ANOVA Searching trials $F_{(1,8)}$=0.7;

p=0.43] or returning trials [RM-ANOVA Returning trials $F_{(1,8)}$=1.5; p=0.26]. However animals with extensive lesions showed significantly fewer VTEs than shams during searching trials (Figure 5c) [Group $F_{(1,6)}$=7.6; p=0.03] but there was no difference between the groups during returning trials (Figure 5d) [Group $F_{(1,6)}$=2.8; p=0.14].

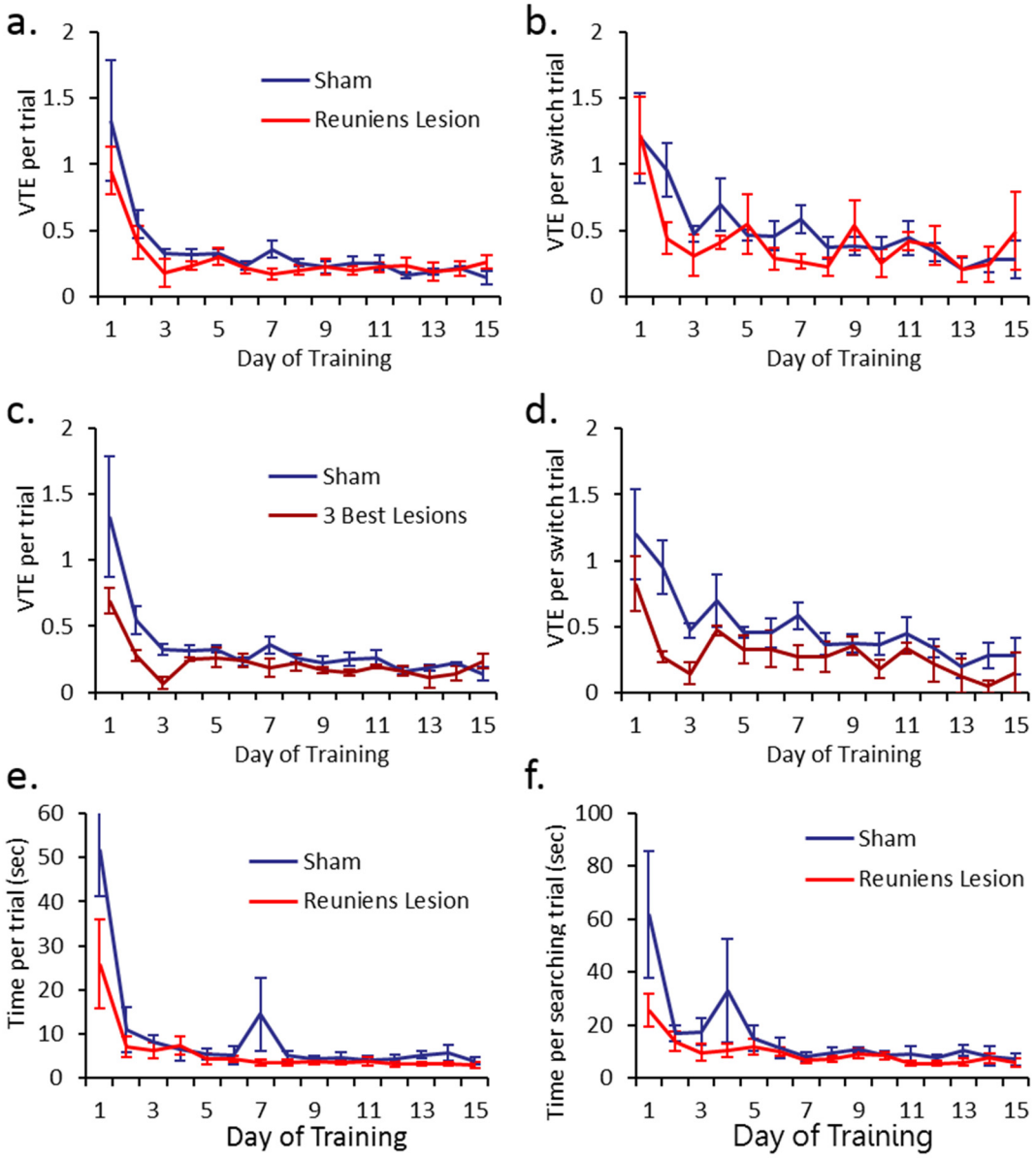


Figure 5: Measures of deliberation or hesitation a) mean VTEs per trial across the whole session. b) mean VTEs per trial during switching trials. c) mean VTEs per trial across the whole session from the most extensive lesion group. d) mean VTEs per trial during switching trials from the most extensive lesion group. e) mean time per trial across the whole session. f) time per trial during initial searching trials (All error bars display SEM)

There was no overall effect on the time per trial (Figure 5e) [Group: $F_{(1,8)}$=1.9; p=0.21, Group*Day $F_{(14,112)}$=1.2; p=0.27]. However lesion rats had shorter searching trials during the first block of the day (Figure 5f) [RM-ANOVA $F_{(1,8)}$=4.7; p=0.06]. This was most obvious for the first four days of testing which correspond to the trials on which the control rats showed increased VTE, after which no difference was seen. This suggests that nucleus reuniens rats may be more impulsive, since the difference is only present during the initial searching trials when sham rats show more VTE.

In summary, nucleus reuniens lesions did not impair acquisition or performance of the serial reversal task. Neither the win-stay nor lose-shift components of the task were affected. However, there were subtle decreases in the amount of VTE and trial length during searching trials, suggesting that lesion rats were more impulsive on trials in which control rats usually show more deliberation due to uncertainty.

3.3.3 Delayed Alternation

Rats were then tested on a delayed alternation task in which the delay between the sample and the choice trial varied between 15 seconds, 1 minute and 5 minutes. Reuniens lesions had no effect on performance on this task at any delay length (Figure 6) [RM-ANOVA (Group) $F_{(1,8)}$=2.9; p=0.13]. There was however an effect of delay [$F_{(1,2)}$=6.4; p=0.01] showing that animals in both groups performed better at shorter delays. However at every delay, animals in both groups performed significantly above chance [t-test against 50% p<0.05 for all delay/group combinations].

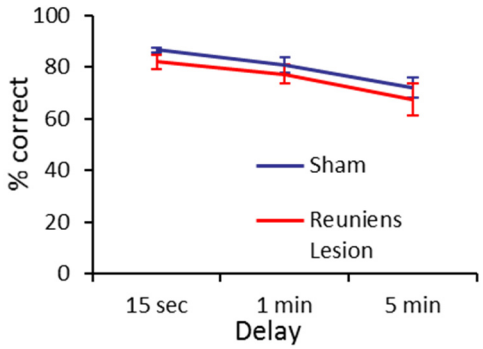


Figure 6. Percent correct alternations at each delayed length (+/- SEM)

3.3.4 Water Maze Reference Memory and Reversal

Rats were then tested on a reference memory task in the water maze. The latencies and path length to reach the platform were recorded for each trial. Since there was no difference in swimming speed [RM-ANOVA $F_{(1,8)}$=0.3; p=0.62] the latencies were

used as a measure of learning as they were less affected than the path lengths by errors in the video tracking software. Lesion rats showed an initial deficit which decreased as the task was learned (Figure 7a) [RM-ANOVA Group $F_{(1,8)}$=2.4; p=0.16; Group*Day $F_{(5,40)}$=2.5; p=0.05], although animals in both groups showed learning over

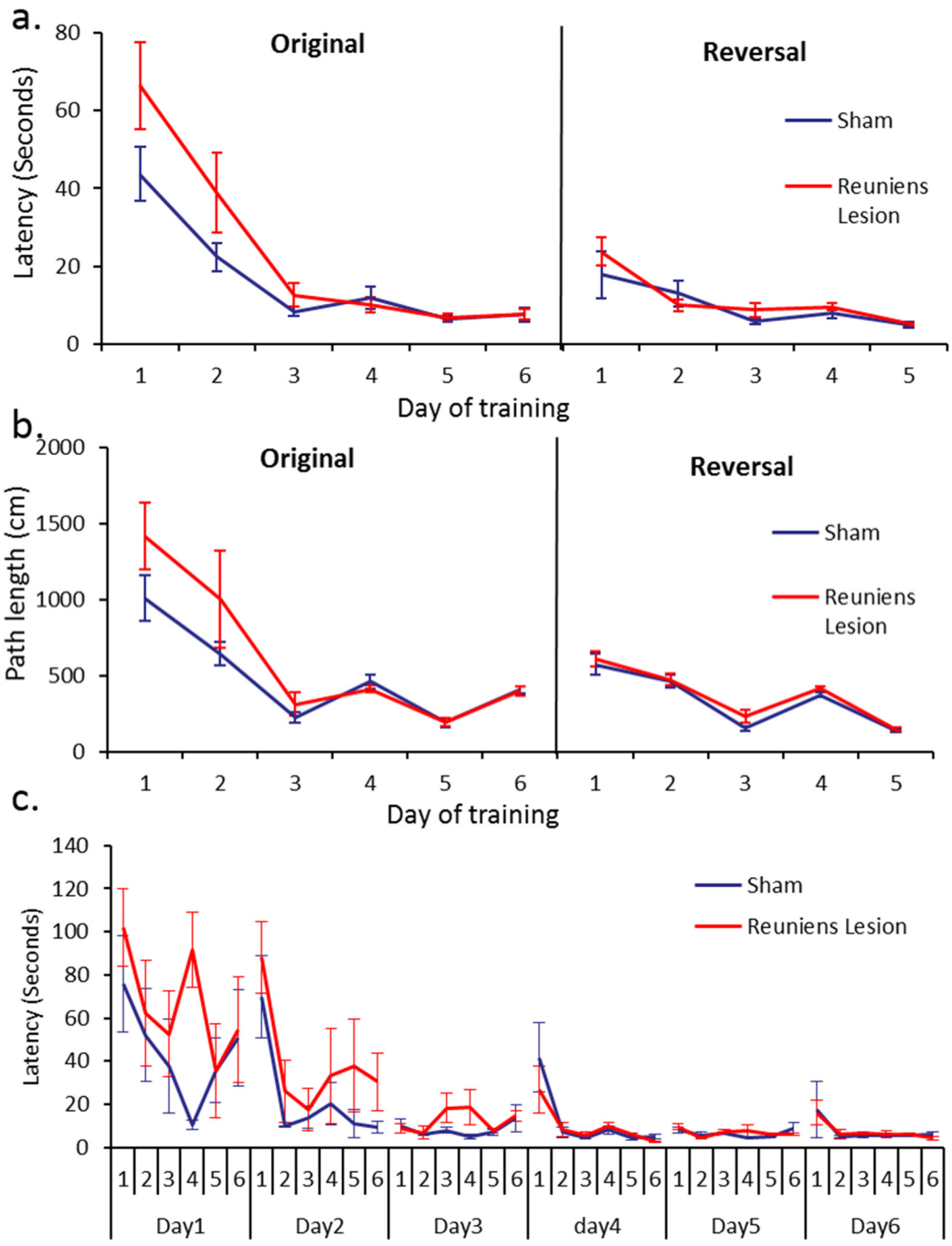


Figure 7: Watermaze performance a) mean latency to find platform (+/- SEM) b) mean path length to platform (+/- SEM) c) latency per trial (+/- SEM)

days [Day: $F_{(5,40)} = 34.8$; $p<0.001$]. The path lengths were also analysed and showed the same trends as the latencies although with more variability (Figure 7b).

During the probe trials, lesion animals appeared to show a lower proportion of time in the correct quadrant compared to control animals, however statistically there was no overall effect on dwell time in the correct quadrant (Figure 8a) [RM-ANOVA Group: $F_{(1,8)}=1.4$; $p=0.28$] although on the first probe trial, rats in the lesion group showed slightly reduced dwell time in the correct quadrant compared to shams. This effect neared significance [t-test: $t_{(8)}=-2.1$; $p=0.06$]. Animals in both groups improved across days [RM-ANOVA Day: $F_{(2,16)}=15.2$; $p<0.001$], although both groups only reached a performance significant above chance on the day 6 probe trial [1-way t-test with Bonferroni correction; Shams: $p=0.023$ Lesions: $p=0.033$]. Following the 6th day of training, animals were given a probe 48 hours later (Figure 8a). There was no significant difference in dwell time in the target quadrant [t-test $p=0.61$].

Sessions were also analysed at the trial-by-trial level (Figure 7c). No differences were seen between groups although, as would be expected, there was a trial effect [RM-ANOVA $F_{(5,40)}=12$; $p<0.001$] suggesting that animals in both groups showed learning throughout the session, and that this was most prevalent on early days of training [RM-ANOVA Day*Trial interaction $F_{(25,200)}=2.1$; $p=0.003$].

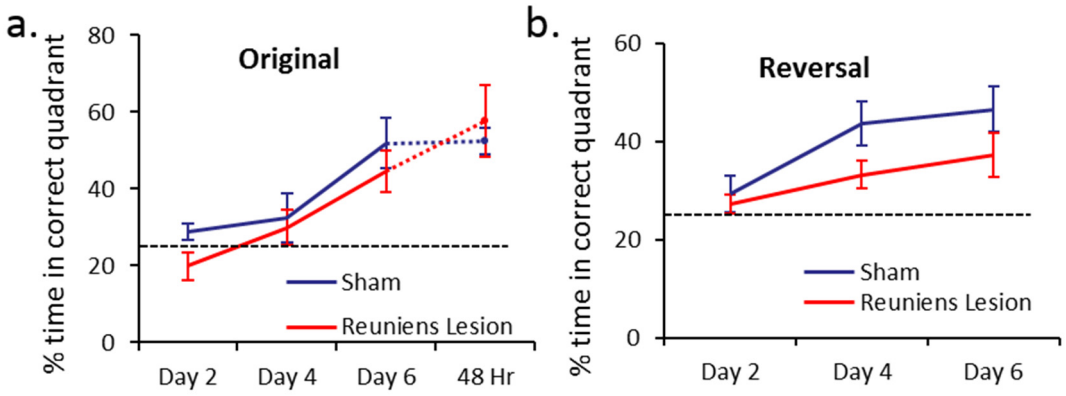


Figure 8: Water maze probe trials: percent time in correct quadrant during a) original platform training and b) during platform reversal. Error bars display SEM

Upon switching the platform location to the opposite quadrant of the water maze, there were no differences seen between the groups (Figure 7a) [RM-ANOVA Group: $F_{(1,8)}$=0.6; p=0.48 Group*Day $F_{(5,32)}$=0.9; p=0.47]. Again, probe trials were conducted on every second day and no differences in dwell time were observed (Figure 8b) [RM-ANOVA $F_{(1,8)}$=2.6; p=0.15]. Animals in both groups improved across days [RM-ANOVA $F_{(2,16)}$=15.1; p<0.001], and both groups reached significance above chance on day 4 [1-way t-test with Bonferroni correction Shams: p=0.02 Lesions: p=0.03].

In summary, reuniens lesion rats were able to learn the reference memory task and perform in probe trials as well as shams, although there was a slight initial impairment on the first few days of training seen in both the trial latencies and the first probe test. Following platform reversal, both groups performed equally well suggesting no deficit in flexibility.

3.3.5 Strategy switching

Rats were then trained on a strategy-switching task on the plus maze. For this experiment one lesion animal had to be excluded because of seizures, leaving the lesion group at only 4 animals. Consequently the following results may be somewhat underpowered. Rats were trained on each strategy until they reached a criterion performance of 10 consecutive correct trials. Performance was measured as number of trials to reach the criterion performance. Figure 9 shows the average number of trials taken to reach the criterion performance on each strategy. An interesting effect is seen for both the first response and the response reversal. Lesion animals required fewer trials (around 33% fewer) to reach the criterion. An RM-ANOVA with strategy-type and within-strategy-reversal as within-subject measures was performed. Both group [$F_{(1,7)}$ = 4.4; p=0.07] and Group*Strategy interaction [$F_{(1,7)}$=4.9; p=0.06] were very close to reaching significance. As can be seen in Figure 9, lesion rats reached criterion performance faster than controls for both of the response strategies [t-test Response 1: p=0.19; Response 2: p=0.03], but there was no difference seen in performance on the place strategies [t-test Place 1: p=0.43; Place 2: p=0.91].

In summary, nucleus reuniens lesions appear to facilitate learning new egocentric response strategies but have no effect on switching strategies or on learning allocentric place strategies. Lesions also have no effect on reversals within a strategy.

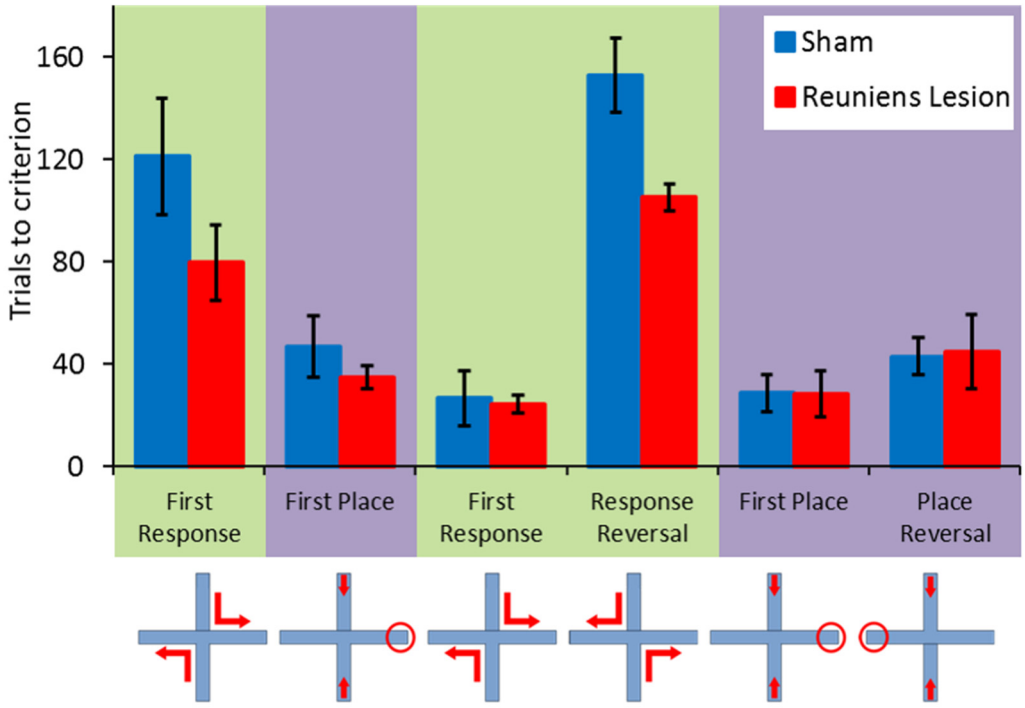


Figure 9: Performance in the strategy switching task: trials to criterion of 10 consecutive correct trials. Error bars display SEM. Green indicates a response strategy and purple a place strategy

3.4 Discussion

The experiments described here investigate the role of the nucleus reuniens in tasks that have been shown previously to depend upon an intact hippocampus or prefrontal cortex and hence might require communication between the two areas.

3.4.1 The nucleus reuniens is not necessary for acquisition or performance of the serial-reversal task on the double-Y-maze

The double-Y-maze serial-reversal task was hypothesised to require the nucleus reuniens because nucleus reuniens lesions have been shown to reduce trajectory dependent activity in the CA1 region of the hippocampus (Ito *et al*. 2015), and it is likely that in a hippocampus-dependent task, trajectory dependent activity might be the mechanism by which a trajectory based task is learned (Stevenson, 2011). The results obtained here reveal that neither acquisition nor performance of the double-Y-maze serial-reversal task were impaired by lesions to the nucleus reuniens. This result suggests one of two possibilities. Either lesions to the nucleus reuniens do not reduce trajectory dependent activity in CA1 in this task, or they do reduce trajectory dependent activity, but the ability to learn and perform the task is independent of CA1 trajectory dependent activity. If the former is true, it would suggest that trajectory dependent activity in CA1 can have different origins depending on the demands of the task. If the latter is true, it would suggest that although the onset of trajectory dependent activity correlates exactly with the time-point at which the task rules are learned, as well as only occurring when there is a task (Stevenson 2011), trajectory dependent activity, at least that seen in CA1, is a secondary effect of learning the task rather than reflecting the memory itself.

The data from Ito *et al. (*2015) would seem to suggest that the second option is more likely since they showed that nucleus reuniens lesions reduced trajectory dependent activity in CA1. However, in their experiment, they used a continuous T-maze alternation task which is not hippocampus-dependent, whereas the serial reversal task used here is hippocampus-dependent. In addition they also found lower levels of trajectory dependent activity in CA3 than in CA1. In contrast, in the previous chapter we found similar levels of trajectory dependent activity in CA1 and CA3 in the double-

Y-maze serial reversal task used. Taken together with the results from this experiment, these findings may suggest that the trajectory dependent activity seen in CA1 in the double-Y-maze task is different from that seen in the continuous-T-maze task used by Ito *et al*. The memory demand in the two tasks is different; in the continuous alternation task, the rat needs to remember the past location temporarily until it can make a choice of the next location on a trial-by-trial timescale, whereas in the double-Y-maze serial reversal task the goals are rewarded in blocks which might allow the hippocampus to represent each block as a separate 'episode' by rate-remapping. Since the double-Y-maze is hippocampus-dependent, trajectory dependent activity in this task may either be generated in CA3, or require the CA3 to CA1 projections, in contrast to the hippocampus-independent T-maze task which might only involve the reuniens inputs to CA1.

Testing this hypothesis would require recording from both CA3 and CA1 from animals with nucleus reuniens lesions while they learned this task. If nucleus reuniens caused no difference in trajectory dependent activity in the serial reversal double-Y-maze task then it would suggest that trajectory dependent activity originates in different areas depending on the demands of the task. Alternatively, if nucleus reuniens lesions did cause a reduction in CA1 trajectory dependent activity, then it would suggest that trajectory dependent activity is not necessary for supporting task memory. However, another possible outcome would be a reduction in CA1 trajectory dependent activity without change to CA3 trajectory dependent activity. This would suggest that CA3 may support learning of the task.

3.4.2 The nucleus reuniens is not necessary for the delayed alternation task on the Y-maze

Our results suggest that the reuniens is not involved in solving the delayed alternation task on the Y-maze, or that in this task other brain areas may be able to compensate so that no deficits are seen. Since this task should be both mPFC and hippocampus-dependent (Shaw and Aggleton 1993, Dudchenko *et al.* 2000), the result appears inconsistent with the previous results that suggested the nucleus reuniens is necessary for spatial working memory tasks specifically when the task requires both the mPFC

and the hippocampus (Hembrook and Mair 2011, Hembrook *et al.* 2012). This suggests that deficits following nucleus reuniens lesions may be very dependent upon the maze, training or pre-training used.

The most directly comparable study investigated the role of the nucleus reuniens in versions of delayed non matching, contrasting the effects on the varying choice delayed non-matching task on the radial maze (VC-DNM) to the effects on a delayed non-matching to position (DNMTP) task. The DNMTP task could be solved egocentrically while the VC-DNM task had an allocentric component, but both involved selecting from two options the one that was not previously sampled (Hembrook *et al.* 2012). In the VC-DNM task, animals start in the sample arm and then travel to the holding arm in which they are delayed for a set time, they then must choose between the sample arm and another randomly open arm which contains a reward. The task is run continuously with the rewarded arm becoming the sample arm for the next. This task has the same basic rule as the delayed alternation task used here on the Y maze, but with two important differences; it has an allocentric component, as the correct arm could be on either side of the sample arm preventing the use of a purely egocentric strategy, and also, unlike the task in the Y-maze, the arms used are not repeated every trial, which might reduce the effect of interference. Surprisingly, the VC-DNM task is not impaired by nucleus reuniens lesions while the DNMTP task was impaired.

Despite the similarities between the tasks, the VC-DNM task is hippocampus-dependent but not mPFC-dependent (Porter *et al.* 2000), while a version of the task in which the same two arms are used for the whole experiment (and the same task on the T maze) has been shown to require both the mPFC and the hippocampus (Porter *et al.* 2009, Shaw and Aggleton 1993, Dudchenko *et al.* 2000). Unless changing the shape of the maze from a T-maze to a Y-maze changes the brain areas involved (which seems unlikely), the task used in this experiment is mPFC and hippocampus-dependent and should therefore require the nucleus reuniens according to the hypothesis suggested previously. Our result contradicts this.

One possible explanation for this anomalous result is that it is the effect of prior training of a different task on the maze. Porter *et al.* (2000) showed that although the repeated choice (RC-DNM) task in which the choice arms were kept constant throughout training (rather than varying each trial) was highly dependent upon both the mPFC and hippocampus, pretraining on the VC-DNM task resulted in the RC-DNM task becoming mPFC-independent (although it remained hippocampus-dependent). This suggests that the RC-DNM task is normally dependent upon the nucleus reuniens but that following training on the varying choice version of the task, the maze or task representation changes and is no longer dependent upon the mPFC. This would then remove the need for the connection via the nucleus reuniens. The Y-maze delayed alternation task is effectively identical to the RC-DNM task and therefore would be expected show similar patterns of mPFC, hippocampus and reuniens dependence. Since the animals in this experiment had already learned a different task on the same maze (although a more complex version of the maze), they may have developed a representation of the maze that did not require this interplay between the mPFC and hippocampus. The double-Y-maze task is hippocampus dependent (Ainge *et al.* 2007), but has not been tested for mPFC-dependence, although the results reported here suggest that it is not, or at least suggests that it does not require the mPFC to communicate with the hippocampus via the nucleus reuniens. It therefore seems possible that this prior learning may have influenced the animals to generate a different representation of the maze or task and therefore remove the dependence upon the mPFC and nucleus reuniens. Based on the results described in the previous chapter, it is tempting to speculate that this 'different representation of the maze' might in fact be the trajectory dependent activity which develops in both CA3 and CA1 during acquisition of the double-Y-maze serial reversal task. Further research is therefore needed to determine whether the Y-maze delayed alternation task is truly nucleus reuniens-independent, or whether the negative result reported here is merely an effect of prior learning on the maze.

3.4.3 The nucleus reuniens may be involved in deduction or selection of allocentric strategies

We saw a slight impairment during the initial place learning in the watermaze. This was not observed in any of the previous lesion studies (Dolleman-van der Weel *et al.* 2009, Loureiro *et al.* 2012). This deficit could be because lesion animals have a deficit in learning or performing allocentric navigation, however this is unlikely because animals reached the same level of performance as the control group and were unimpaired during reversal learning. Rather it suggests either that reuniens animals were initially attempting to use another strategy, or were initially impaired at learning the rules of the task (for example realizing that the platform was always in the same place) but that once they had learned them, they were unimpaired at flexibly applying them to a new location. It is conceivable that learning the task rules might require mPFC to hippocampus communication, and this might then explain the initial deficit. Reuniens lesions did not significantly affect performance in the reversal suggesting that it is not needed for flexibility within a spatial strategy. Supporting this idea, mPFC lesions have been found to cause no deficits on watermaze reference memory or reversal (de Bruin *et al.* 2001).

The data from the plus maze is somewhat surprising, because the lesion animals did not show a deficit in allocentric strategy learning, which would be expected since it has been suggested that to learn an allocentric strategy requires communication between the prefrontal cortex and hippocampus. However, since the lesion group showed an increased ability to learn an egocentric strategy this again suggests a role for the reuniens in strategy selection. Nucleus reuniens lesions may disrupt the pathway by which the prefrontal cortex predicts the result of possible actions based on spatial information from the hippocampus (Ito *et al*. 2015). The mPFC might provide a representation of the goal and the hippocampus the representation of how to get there. In a scenario where this relay has been broken, this pathway would not function, and so the animal would be more likely to notice that an egocentric strategy might work.

The data from the watermaze reference memory task and the plus maze strategy switching tasks and the previous one are in some ways consistent with the results from

the watermaze and double H-maze place task used by Cholvin *et al*. (2013). This task involves training animals to navigate from one of two locations to a constant end location. After training, probe trials are run from a new location. If animals navigate to the previously trained goal location it implies that they were using an allocentric strategy, while if they follow the sequence of turns that would have been correct from the training arm it implies that they were using an egocentric strategy. The authors found no deficit following reuniens inactivation on the watermaze allocentric task but did find an impairment on switching to allocentric navigation in the double-H maze. They suggest that this difference is because in the double-H maze, control animals initially learn the task as an egocentric series of responses but are capable of shifting to a place strategy when the egocentric strategy fails. They suggest that this shift is where the impairment following nucleus reuniens inactivation arises. Since watermaze training never involves repeated training from the same start location there is never any reinforcement of a response strategy so this deficit would be less obvious. In our strategy shifting task, there was never any testing of the allocentric strategy being truly remembered as an allocentric place. On our plus-maze, but not the double-H-maze, the allocentric task could possibly be solved by a place-specific response strategy in the same way that the rats in the H maze learned both of the pretrained routes to the goal arm egocentrically, and relied this egocentric strategy in preference to an allocentric strategy. This might still require the hippocampus to communicate with the prefrontal cortex in order for the animal to know its starting location, however communication back to the hippocampus would be unnecessary since the actual navigation as a response would be dependent on the striatum. In some ways, the result that lesion animals are faster at learning the response strategy on the plus maze is consistent with the data from the double H maze (Cholvin *et al*. 2012), in that both show an increased use of egocentric strategies.

Collectively these results suggest that the nucleus reuniens does not have a functional role in spatial tasks separate from its position as a relay between the mPFC and the hippocampus. Data from multiple experiments however suggest that this pathway of

information can be very necessary for certain types of task, which require interaction between the mPFC and hippocampus.

3.4.4 Limitations and future work

There are several limitations to our methods which must be considered when interpreting our results. Firstly we used lesions to test the function of the nucleus reuniens. This method allows the possibility that remaining brain tissue either within the nucleus reuniens or other areas might compensate. This would have a particular effect on the later tasks we used because they would have had more time to develop compensatory strategies particularly since they were being trained on other spatial tasks during this time. However, since we saw no deficit on the first task, which we trained immediately after the lesions would have occurred, and did see deficits in later tasks, this explanation seems an unlikely reason for the negative result seen in the first experiment. However this experiment could be improved by using pharmacological or optogenetic inactivation to acutely disrupt the nucleus reuniens reducing any compensatory effects.

A second problem is that our lesions were not complete. Two of the lesions only encompassed 50% of the nucleus reuniens and while the other three were more extensive none were complete. It is possible that the reason for the negative results seen in some of the experiments is that the remaining tissue was sufficient to support the behavioural tasks tested. However, since we saw no deficit on any performance measures even in the most extensively lesioned animals (one of whom had an 83% lesion) this seems unlikely to explain the double-Y-maze data.

Another potential problem with the design of our experiment is the sequence in which the tasks were trained. This could possibly cause interference, or have influenced the strategies used by the animals in the later tasks. This is a possible explanation for why we saw no deficit in the working memory task on the Y maze, since animals might have already formed a representation of the maze during acquisition of the double-Y-maze task which might allow them to solve the working memory task using the hippocampus but not the mPFC (Porter *et al.* 2000). The sequence effect is also a

possible contributor to the enhanced ability to notice and learn an egocentric strategy on the plus maze. Since rats had previously been trained in tasks which could be solved either allocentrically or egocentrically, and the lesioned rats were potentially slightly impaired at allocentric navigation, they may have relied more on egocentric strategies previously which might explain their enhanced learning of the plus-maze response strategy compared to control animals who might have relied on allocentric navigation more in previous tasks. This could be tested by repeating the later experiments with naïve animals. If the positive effect of reuniens lesions is merely an artefact caused by prior learning then it would not be seen in naïve animals.

Since it is clear that reuniens lesioned animals are capable of learning both egocentric and allocentric navigational strategies, and can even be unimpaired on forced strategy switching, the contribution of the nucleus reuniens must be more subtle. One possibility which has not been tested here is that rather than affecting 'forced strategy' switching, it changes the animal's preferred strategy. One possible method to test this would be to use a task which can be solved using multiple strategies, rather than rewarding a specific strategy but not the other as was done here. When animals are trained with one start location and one end location on the plus maze (allowing the possibility of using either a response or a place strategy), they typically rely on a place strategy during early days of training but after many days of training shift to using a response strategy and this can be tested using a probe trial from a new start location (Packard and McGaugh 1996). Similarly the starmaze can be used to test whether animals use an egocentric sequence of body turns or an allocentric place memory (Rondi-Reig *et al.* 2006). When control mice are trained to navigate between two fixed locations on the starmaze, they normally learn both an egocentric body-turn sequence strategy and an allocentric place strategy, and in subsequent trials starting in new locations they switch between the two strategies without reinforcement for doing so (Rondi-Reig *et al.* 2006*)*. If the nucleus reuniens changes an animal's preference for one type of strategy, for example egocentric strategies, then all nucleus reuniens animals might show an increased tendency to use one of the two possible strategies. Conversely, if the reuniens is involved in switching between strategies, then reuniens

animals as a group may show no preference for a specific strategy, but having once developed a strategy each animal would show no unforced switching to the other strategy.

In conclusion, this study has contributed to the understanding of the role of the nucleus reuniens in navigation. It suggests that the nucleus reuniens may play a role in the deduction or selection of allocentric strategies. This may explain its role as part of a pathway between the prefrontal cortex and hippocampus. In addition, the surprising result from the double-Y-maze study, taken together with the result from the previous chapter, suggests that trajectory dependent activity may not be one unique phenomenon, but actually reflect different inputs and functions depending on the specific demands of the task.

4 The contribution of the MEC to CA1 place cells

4.1 Introduction

The medial entorhinal cortex (MEC) is thought to be the major spatial input to the hippocampus. Most cortical inputs to the hippocampus come via either the medial or lateral entorhinal cortices and the MEC is thought to provide the spatial information with the LEC providing contextual information (Kerr *et al.* 2007). The MEC input consists of several different spatially modulated cell types, with some neurons encoding head direction, boundaries, or location (grid cells) (Taube 2007,Solstad *et al.* 2008, Fhyn *et al.* 2004), and it is known that the projections to the hippocampus contain all of these different spatial signals(Zhang *et al.* 2013, Sun *et al.* 2015). Recently a major question has been whether the precise spatial activity seen in place cells was the result of summation of inputs from the MEC grid cells.

At first glance, this seems logical, as it has been shown that the different sizes of grid cell lattice can summate to form place field-like activity in several different computational models (Fuhs & Touretzky 2006, Rolls *et al.* 2006, Solstad *et al.*2006, Savelli & Knierim, 2010, Lyttle *et al.* 2013 and others). Anatomical and electrophysiological evidence also appears to support the idea, as grid cells have been recorded from both of the superficial layers of the MEC (Hafting *et al.* 2005, Sargolini *et al.* 2006) that project directly to place cells (Van Strien *et al.* 2009), and in a recent paper, MEC neurons retrogradely labelled from the hippocampus were shown to be grid cells (Zhang *et al.* 2013). However, there is also evidence that grid cell activity specifically is not required for place cell activity; a study looking at the development of place cell, head-direction cell and grid cell activity in the developing rat found that head-direction-modulated activity developed first, followed by border-modulated activity and place-field activity together, with grid-field activity developing a few days later (Langston *et al.* 2010).

The above data suggest that the grid cell input is not necessary for place field formation, but there are also other spatially modulated inputs from MEC, the head direction cells and border cells. Might they be a necessary input? Lesion studies

suggest that while these spatial cells project to place cells, they are unlikely to be necessary for place field generation. Lesions to the MEC do not result in a complete loss of place cell activity (Miller and Best 1980,Brun *et al.* 2008, Van Cauter *et al.* 2008, Hales *et al.* 2014), although they do in general result in a decrease in the spatial precision of place cells (Brun *et al.* 2007, Hales *et al.* 2014), their stability (Brun *et al.* 2007, Van Cauter *et al.* 2008, Hales *et al.* 2014), and in some cases a reduction in the firing rate of place cells (Van Cauter *et al.* 2008, Hales *et al.* 2014). This suggests that the MEC input is not required for place field firing, but rather modulates established place fields, possibly to refine their spatial precision or to increase their stability. Since the LEC and subiculum also project to the hippocampus and both are known to contain spatially modulated cells it is likely that place cells form fields in response to multiple inputs, and removing one input therefore has little effect on the existence of place cell activity in the hippocampus. Together, these results suggest that place cell activity is not dependent upon the inputs from the MEC. So what does this input contribute if place cell activity can form without it being there at all?

The first paper looking at the effect of entorhinal lesions on place cell activity was published in 1980. In it, Miller and Best provide evidence that following lesions to the entire entorhinal cortex, place cells in rats exploring a radial arm maze which is rotated within a room become anchored to local cues within the maze, contrasting with control animals whose place fields are anchored to the extramaze cues in the room. Recent electrophysiological recordings from both the MEC and LEC suggest that the MEC particularly may be providing this distal cue information (Neunuebel *et al.* 2013). Rats ran laps around an annular maze in which different floor textures provided strong proximal cues, and several distinct landmarks on the walls provided distal cues. MEC neuronal activity rotated with the global reference frame based on the distal landmarks, while LEC activity rotated with the local reference frame.

A closer inspection of the data from Hales *et al.* (2014) provides other evidence in support of the idea that the MEC contributes distal cue information. The authors trained rats for 6 days with one configuration of the watermaze, and then changed all the cues, as well as the geometry of the room before performing what they call

"reversal" training. The MEC lesion rats showed a greater impairment at learning this new location; but they also showed high perseveration to the old location. Since all the distal cues, including the room geometry were different, this strongly suggests that these rats were never using these distal cues in the first place but had learned a different strategy using whatever minimal local cues were present within the watermaze. As expected, the control animals show no such perseveration to the old location which makes sense if they originally learned the first platform's location in relation to the distal cues around the room.

This suggests that the reason major deficits in place cell spatial activity have not been seen in previous studies, is not that the MEC isn't contributing necessary information to the place cells, but that another input such as the LEC could contribute enough information from proximal objects (Scaplen *et al.*2014) and odour cues to allow the stabilization of the spatial place cell map (Zhang and Manahan-Vaughan 2015 but see Aikath *et al.* 2014).

The purpose of this experiment was to test whether animals with MEC lesions use distal cues to orient their place cells within an environment. In addition, it tests the hypothesis that place fields in MEC lesioned animals can be anchored to proximal cues. If this is true would imply that in normal environments, place fields can be tied to odour cues or any other available proximal cues allowing the largely unaffected place field firing seen in previous papers.

The standard method to test whether spatially modulated cells are anchored to particular landmarks is to perform 'cue rotation' experiments (O'Keefe and Conway 1978, Muller and Kubie 1987, Chien *et al.* 2014). In these experiments most cues or sources of directional information are removed, leaving certain controlled cues which can be rotated coherently around the environment. The extent to which place fields follow the rotating cues can then be found by comparing the field locations during the different sessions. These experiments have been performed using both distal cues, which are predominantly visual, and proximal cues, which usually have visual, tactile and olfactory components. These experiments have revealed that place cells are

preferentially anchored to distal cues, but may be controlled by proximal cues, such as objects, particularly when they are familiar or are the only cues available (Gothard *et al.* 1996, Cressant *et al.* 2002, Scaplen et al 2014).

The current experiment was designed to test the effect on place field location of performing 90° cue rotations, first of visual distal cues in the absence of proximal cues, and then rotating proximal 'object' cues in the absence of any distal cues. Other potential polarising cues were removed or masked as much as possible to reduce their availability as a source of directional information. In addition, the distal or proximal cues used were unambiguously distal or proximal, as the distal cues were purely visual cues which are out of reach of the animal, and the proximal cues were three dimensional objects which the animal could explore from all directions because they were not at the extreme edge of the environment.

This experiment was carried out using mice, rather than rats, because of the range of genetic techniques available for silencing or modulating neuronal activity in specific populations of cells. Place cells have been recorded in mice, but mouse place fields show reduced stability over time compared with rats, particularly when mice are not engaged in performing a spatial task (Kentros *et al.* 2004). As a preliminary experiment, ibotenic acid was used to lesion the MEC to establish whether the MEC as a whole has a role in distal cue processing. This will allow any results to be followed up using more specific genetically targeted silencing techniques.

4.2 Methods

4.2.1 Subjects

Subjects were 8 male C57Bl6 mice aged around 8 weeks at the start of the experiment. An additional 4 mice underwent electrode implantation but no single-units were identified during screening so they were not included in the experiment. Following surgery, mice were housed individually on a 12hr light/dark cycle and had free access to food and water in the home cage. All unit-recording occurred during the light phase of the cycle. All procedures complied with the Animals (Scientific Procedures) Act 1986.

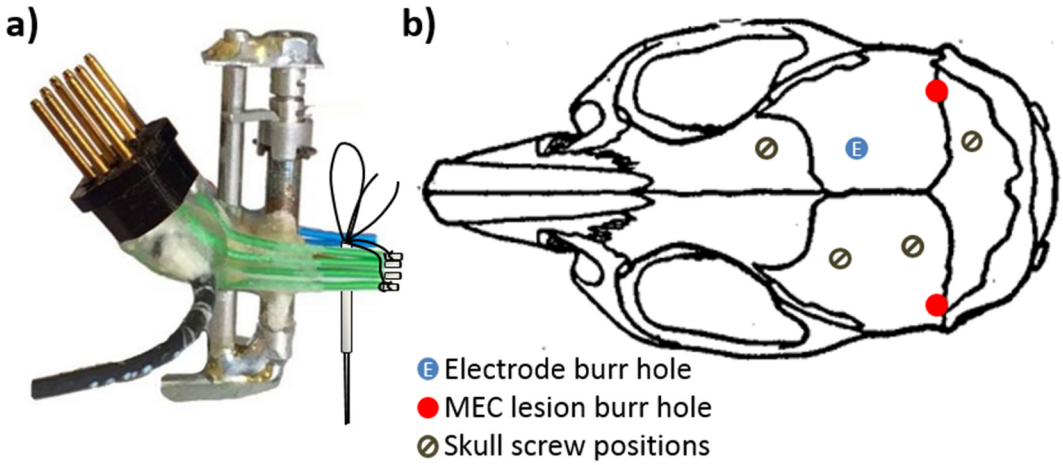


Figure 1: a) Axona microdrive with 2 tetrodes b) positions of the electrode, lesion burr holes and skull screw positions on the skull. The burr holes were located between the suture and the transverse sinus and the injection pipette was inserted just in front of the sinus at the posterior side of the burr hole. 4 skull screws were inserted into the skull and with dental cement formed the base of the microdrive.

4.2.2 Micro-drive implants

Two-tetrode microdrives (Axona Ltd, UK) were used for all implants. These reusable drives consist of a steel frame and drive screw, connected with dental cement to a cannula containing the electrodes, a ground wire, and an Axona plastic connector (Figure 1a).

Tetrodes were constructed from 17 µm HML-coated platinum(90%)-iridium(10%) wire (California Fine Wire, CA). 4 lengths of wire were twisted and heat-annealed together using a heat gun at 240°C for ~6 seconds to form each tetrode. 2 tetrodes were then loaded together into the drive cannula.

The insulation was burned off ~3 mm of each wire of each tetrode and they were connected individually to the Axona connector wires. A good connection was achieved by wrapping the uninsulated portion of the tetrode wire around the connector wire several times and then covering the join with silver conductive paint (Electrolube, UK). The connections were then insulated and protected by applying nail-varnish to completely cover the connector wires and tetrode wires all the way to the cannula.

Shortly before surgery, the two tetrodes were trimmed using ceramic scissors (Fine Science Tools, Germany) under a microscope to ~2 mm longer than the base of the

drive. The electrode tip was inspected under the microscope to check that all electrode tips were circular and roughly equal in length. The tetrode was then plated with gold solution (Neuralynx, MT) as described in Section 2.2.2. A protective 18 gauge outer cannula was then placed around the drive cannula and held in place with sterile Vaseline.

4.2.3 Surgical Procedures

Of the 8 mice used in this experiment, 4 received bilateral MEC lesions and an microdrive implant and 4 received sham lesions and a microdrive implant. The mice were randomly assigned to a group and both the recording sessions and initial clustering analysis were performed with the experimenter blind to experimental group.

The lesions/sham lesions and electrode implants were performed during the same surgery which took between 3 and 5 hours. Mice were anaesthetized using isofluorane gas (Abbott Laboratories, IL) in oxygen. Analgesia was achieved by subcutaneous administration of small animal Rimadyl (Pfitzer Ltd, UK) at a dose of 0.08ml/kg bodyweight. A subcutaneous injection of 2.5ml isotonic saline and glucose solution was also administered at this time. The eyes were covered throughout surgery with hydrating eye-gel (Viscotears, TX). The scalp was then shaved and cleaned with antiseptic and the mouse was then fixed into a stereotaxic frame (Kopf, CA) using a bite-bar, nose-cone and two non-traumatic ear-bars. The mouse was placed on a thermostatic heat blanket and covered with a drape.

The skull was exposed via a midline scalp incision, and holes were drilled immediately behind the lambdoid suture (and in front of the transverse sinus) at 3.6 mm on each side of the midline as calculated at Bregma. For all mice a hole was made in dura above the injection site. For the 4 MEC lesion mice, a glass micro-pipette (Drummond Scientific, PA) was lowered into the brain at an angle of 10° forwards in the anterior-posterior plane. The injection site was just anterior to the transverse sinus, and between 3.5-3.7 mm lateral to the midline (Figure 1b). 4 injections of 20nl (2 mice) or 40nl (2 mice) of ibotenic acid (Tocris, UK) (10mg/ml, pH7.4 in PBS) were made at depths of 2.8 mm, 2.3 mm,1.85 mm and 1.4 mm below dura. After each injection, the pipette

was left in place for 5 minutes before being raised to the next injection site. Following injections (or for shams, piercing of the dura only), sterile gelatin sponge (Spongostan Special, Ferrosan A/S, Denmark) soaked in saline, was placed onto the brain surface.

4 screw holes were drilled in the skull; one on the frontal plate, two in the left lateral plate, and one in the occipital plate (Figure 1b). 4 self-tapping stainless-steel 120TPI screws (Antrin Miniature Specialties Inc, CA) were inserted into the holes and held in place with dental cement ((Simplex rapid acrylic denture polymer, Associated Dental Products Ltd, UK). One skull-screw had a grounding wire attached before surgery. The electrode hole was then drilled 2 mm posterior and 2 mm to the right of Bregma (Figure 1b), dura was removed, and the electrode was lowered into position 0.9 mm below dura (~0.15 mm above the CA1 pyramidal layer). The outer cannula was lowered into position around the electrode above the skull, and sterile Vaseline was used to ensure the join was sealed. The ground wire was soldered onto the skull-screw wire and skull-screws, base of the drive and the injection sites were all covered over with more dental cement. Mice were placed on a heat bench at 30°C until they fully regained consciousness and then for a further hour of recovery. They were then given 10 days for recovery, during which all mice regained their presurgery weight, before screening commenced.

4.2.4 Electrophysiological recording

Mice were connected to a 32-channel recording system (Axona Ltd, UK) via a headstage amplifier and pre-amplifier. Screening occurred while mice explored a screening cylinder. The signal was amplified, filtered with a bandpass filter at 600-6000 Hz and single-units were identified using the oscilloscope in the DACQ software (Axona Ltd, UK). If suspected neuronal spikes were observed, a trigger was placed at an appropriate amplitude to collect spikes from putative neurons while minimizing collection of noise spikes. The signal from one wire was duplicated and filtered with a lowpass filter at 300 Hz and a notch filter at 50 Hz to record the local field potential. A camera placed above the environment and an infra-red LED on the headstage amplifier allowed tracking of the mouse's position at a frequency of 10 Hz.

4.2.5 Apparatus

The recording environment was a plastic flowerpot saucer 50 cm across, with a rim 3 cm high and 2 cm wide. This was placed 60 cm off the floor on a stool in the centre of circular curtained enclosure 2 m across. The curtains were navy blue with 6 possible exits at uniform distances around the enclosure and the ceiling was covered with a white sheet to remove any directional cues. A speaker, lightbulb, camera and recording cable were placed directly above the centre of the environment above the white sheet, with a small hole for the camera lens and cable to pass through. During all recordings white noise was played from the speaker to mask any possible directional auditory cues, and all the lights on the outside of the curtains were turned off to reduce any light differences across the environment. For the distal cue sessions, two large distal cues were attached to the curtains with safety pins. One was a white sheet which reached from the floor to the ceiling and was 1.4 m wide, the other was a hula-hoop covered with shiny paper to make a circle 1m in diameter and attached so that the base was level with the height of the saucer. These cues were attached at an angle of 130° to each other and could also be rotated by 90° clockwise around the enclosure (Figure 2a). For the proximal sessions, the distal cues were removed and three proximal cues were placed within the saucer (Figure 2b). They ranged in height from 6-11 cm and were different in shape, colour and texture. They were placed in three locations to form an isosceles triangle at the edge of the floor of the saucer (as in Save *et al.* 2005) but as the saucer had a 2 cm rim the mouse could walk around the outside of them on top of the rim.

4.2.6 Cue rotation sessions

Once multiple place cells had been identified from a screening session, mice were recorded for three days in the environment with distal cues followed by three days in the environment with proximal cues.

On a given day mice would have 4 sessions each of 15 minutes with a break of approximately 5 minutes between sessions. For distal and proximal cue rotation days the order of sessions was: standard 1, standard 2, cue-rotation 90° clockwise, cue-rotation 90° anticlockwise back to standard 3 (Figure 2).

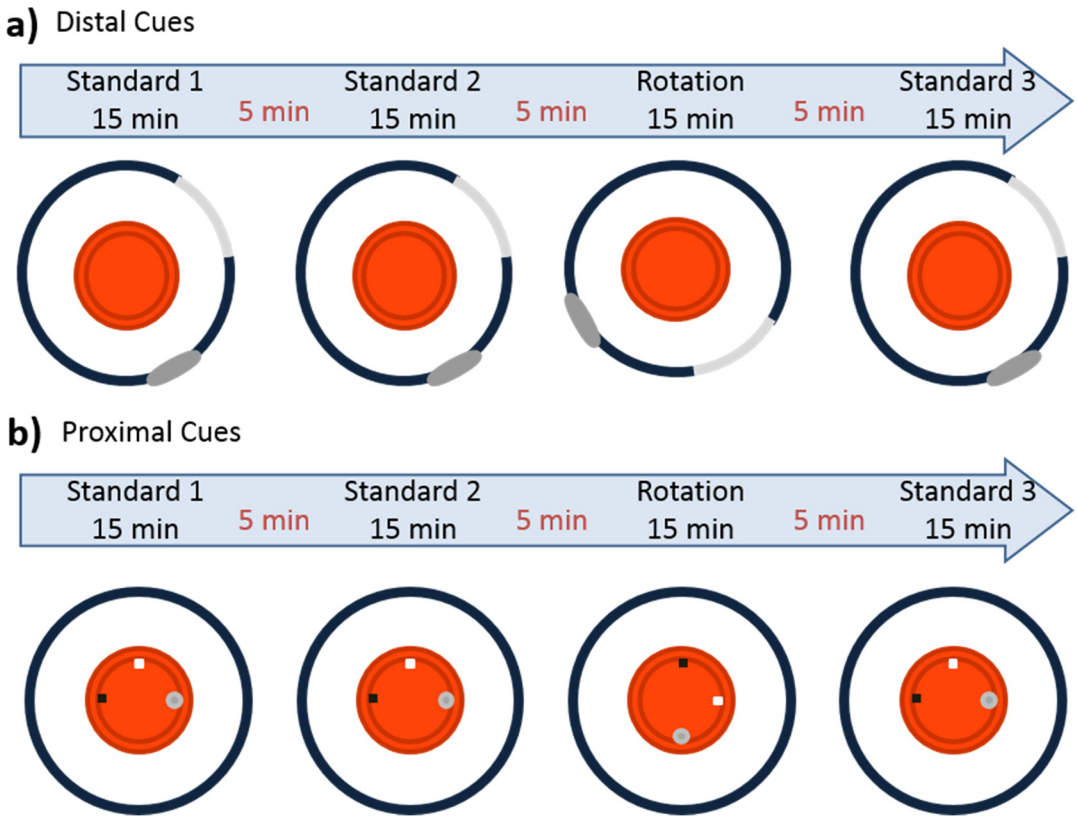


Figure 2: Experimental protocol: place cells were recorded in four successive 15min sessions. Mice were removed for ~5mins between sessions while the environment was cleaned and any necessary cue rotations were performed. a) distal cues consisted of a large white sheet from floor to ceiling and a hula hoop covered with reflective material, arranged approximately 130° from each other b) proximal cues consisted of 3 objects, ranging in height from 6-11 cm and arranged in increments of 90° around the environment

On each session, the mouse inside its home cage was covered with a blanket and carried into the curtained enclosure. The mouse was carried, still covered, between half a turn to 2 turns around the environment in order to prevent the use of vestibular inputs for orientation. The mouse was removed from the home cage, connected to the recording system, and placed in the recording environment. They were then allowed to forage for scattered Cheesy Wotsits crumbs (Walkers, UK) for 15 minute while single-unit and local field potential data were recorded. Following completion of a session, mice were unplugged and placed back into their home cage, which was then covered. The saucer was then sprayed and wiped clean with absolute alcohol and placed back in the same orientation (although cues moved between sessions the floor of the environment did not). Any necessary cue rotations were performed during this

time while the home-cage was covered. The cage was then picked up and carried round the environment to a random location, before the mouse was taken out plugged back in to the system and the next session began.

4.2.7 **Data analysis**

Following data collection by Dacq-USB (Axona Ltd, UK), data files from each session of the day were combined and analysed using a custom Matlab script and a clustering algorithm (KlustaKwik2, developed by Kadir *et al.(*2014)). Clusters identified by the algorithm were then visualized in Klusters (developed by Hazan *et al.* (2006)) so that noise clusters could be deleted and incorrect clustering could be fixed. During visual inspection, clusters whose waveforms appeared very similar were combined and irregular spikes judged to be noise were removed from clusters where possible. A further Matlab script was then used to generate firing-rate maps for each cluster, plot average waveforms for each channel of the tetrode, and calculate overall firing rate, peak firing rate, waveform width, spatial information content, sparsity and isolation-distance for each session separately and for the combined hour of recording.

Clusters were identified as pyramidal neurons if they had a mean firing rate between 0.1-5Hz, and waveform width greater than 250 μs. Clusters that did not meet these criteria were excluded from further analyses. The firing rate, spatial information, and sparsity were compared between the two groups.

The firing rate map was constructed using an algorithm adapted from Leutgeb *et al.*(2007b). The entire area explored by the animal was divided into pixels 2.5x2.5 cm in size. Firing rates were not calculated for a pixel if the animal never came within 5 cm of the centre of the pixel. The average firing rate for each pixel was calculated using every spike recorded, but weighted using a Gaussian kernel such that spikes close to the centre of the pixel had the most effect on the resultant firing rate. The firing rate (FR) of a given pixel with centre position *x* was calculated as follows:

$$\mathrm{FR}(x) = \frac{\sum_{i=1}^{n} g\left(\frac{S_{i-x}}{h}\right)}{\int_0^T g\left(\frac{y(t)-x}{h}\right) dt}$$

where i is every spike from 1 to the total n of spikes. Si is the position of every spike, [0 T] is the total time of the recording period, y(t) represents the animal's position at time t, and h is the smoothing factor of 2.5cm, and g represents a Gaussian kernel with equation: $g(x) = exp\left(\frac{-x^2}{2}\right)$

The overall firing rate was calculated as the total number of spikes within a session divided by the length of the session, while the peak firing rate was calculated as the value of the highest pixel of the firing rate map.

Sparsity was calculated using the following equation: $Sparsity = \sum(P_i * R_i^2)/R^2$, where *i* is the bin number in the firing rate map, P_i is the probability that bin *i* is occupied, R_i is the mean firing rate in bin *i,* and R is the overall firing rate. Sparsity measures in what proportion of the environment explored by the mouse did spikes occur.

Spatial information content (SI) was calculated using the following equation: $SI = \sum_i P_i \left(\frac{R_i}{R}\right) ln\left(\frac{R_i}{R}\right)$, where *i* is the bin number in the firing rate map, P_i is the probability that bin *i* is occupied, R_i is the mean firing rate in bin *i* and R is the overall mean firing rate. Spatial information content is a measure of how much information about location is carried by one spike (Skaggs *et al.* 1993).

In order to analyse cue-rotation and stability of place fields, the separate firing rate maps for each session were analysed in pairs: Standard 1 v. Standard 2, Standard 2 v. Rotation, and Rotation v. Standard 3. For each pair, the two firing rate maps were overlaid and rotated relative to each other in increments of 5°. The Pearson's correlation between the two maps was calculated at each angle of rotation, and the maximum and minimum correlations and angles of best correlation were extracted.

For each pair of sessions the angle of maximum correlation for every cell active in both sessions was calculated, and plotted on a circular histogram. A Watson-Williams F-test was used to test whether the mean angle of best correlation was the same between groups. In addition a V-test was used to test whether the circular distribution

of angle of best correlation was a uniform distribution or whether it showed a distribution with a mean matching the mean angle expected if the place cells were following the cues; 0° for the Standard 1-Standard 2 pair, 90° for the Standard 2-Rotation pair, and 270° for the Rotation-Standard 3 pair. In addition, the number of cells which were only active in one session of the pair, due to either complete remapping or to electrode drift, was also separately calculated.

The maximum and minimum correlations and angle of best correlation were then used to categorise what each place cell did between sessions into the following categories; stable, rotated, remapped, or ambiguous. A cell was categorised as stable if the maximum correlation was greater than 0.5, the minimum correlation was less than 0, and the angle of highest correlation was between 30° and -30°. A cell was categorised as rotating if the maximum correlation was greater than 0.5, the minimum correlation was less than 0, and the angle of highest correlation was between 60° and 120° for clockwise cue rotations, or -60° and -120° for anticlockwise rotations. A cell was categorized as remapping either if it showed an angle of rotation that did not fall within the previously mentioned bands, or if the maximum correlation was less than 0.5. Finally a cell was classed as ambiguous if the maximum correlation was greater than 0.5 and the minimum correlation was greater than zero. This would indicate that the field was rotationally symmetrical, usually because it was located near the centre of the environment, and so never had a negative correlation. As these cells were unable to be used to determine which cues the place cell were following, they were not counted when calculating the proportion of place cells that rotated, remapped or were stable.

For each pair of sessions, every cell that was active in both sessions (with an overall firing rate greater than 0.1Hz) was included in the analysis and was categorised into the above categories. The proportions of cells which remapped, stayed stable or rotated were calculated for the sham group and the lesion group and a Chi-Square test was used to determine whether the distributions differed.

In case lower precision of place fields in the lesion group skewed results, the rotation analysis was repeated after only including cells with a spatial information content above thresholds of either 0.5bits/spike or 0.3bits/spike. Since many of the cells, particularly in the MEC lesion group had lower spatial information, this greatly reduced the number of cells, but still enabled trends to be observed in the data.

In addition, the analyses of firing rate, spatial information, sparsity and rotation angle, were also applied after removing clusters which were identified to be of poor quality. Cluster quality was assessed using a combination of L_{ratio} and isolation distance (ID) (see Schmitzer-Torbert *et al.* 2005 for equations and validation of the method). Both of these measures assess how well isolated a cluster is from other spikes recorded from the tetrodes, either those spikes identified as noise, or spikes within other clusters. Clusters were classed as excellent if ID>30 and L_{ratio}<0.1, good if ID>20 and L_{ratio}<0.15 and acceptable if ID>15 or L_{ratio}<0.2. The values calculated for spatial information, sparsity, firing rate and rotation were recalculated for each tier of cluster quality to identify whether including cells with poor cluster quality had an effect on the results.

4.2.8 Histology

Following completion of data collection, mice were anaesthetized with isofluorane and given a lethal dose of sodium pentobarbitol (Euthatal, Meridal Animal Health, UK). The tissues were fixed by transcardial perfusion of ice-cold 4% paraformaldehyde. The brains were then extracted and stored overnight at 4°C in 4% paraformaldehyde before being cryoprotected in 30% sucrose solution. They were then stored in a -70° freezer. Brains were sectioned in the sagittal plane at 32 μm thickness with a cryostat-microtome. Half of the sections were mounted on polysine slides (Thermo Scientific, UK), stained with 0.1% cresyl-violet, and coverslipped in DPX (Sigma-Aldrich, UK). Sections were then mounted and photographed at 10x magnification using Image-Pro Plus (Media Cybernetics, USA). The area of MEC, ventral presubiculum and ventral hippocampus were then calculated for the control animals by drawing around each region on the micrograph, and using ImageJ (NIH, USA) to measure the area. This was then averaged between the control animals. The total area of spared MEC, ventral

presubiculum and ventral hippocampus was then calculated for each lesion animal and the percentage of tissue lesioned was calculated. To identify spared tissue, the sections were also examined at 20-30x magnification to determine whether spared regions of tissue contained neurons or only glia. If tissue contained any neurons, it was counted as healthy tissue, but if only glia were present it was counted as scar tissue and was not included in the total area of spared tissue.

4.3 Results

4.3.1 Histology

Lesion sizes were variable. The first two MEC lesions were very small, with approximately 30% lesions bilaterally, however they were very well restricted to the MEC with no damage to other structures. The second two animals had much more complete MEC lesions, but these were not well restricted to the MEC. The postsubiculum, dorsal hippocampus and subiculum were spared in both animals, however there was extensive damage to ventral hippocampus and ventral presubiculum, in addition to nearly complete lesions of the MEC. Consequently, it is possible that results observed in these animals may be the result of damage to additional structures. However, the MEC was nearly completely lesioned with only a

	Lesion 1		Lesion 2		Lesion 3		Lesion 4	
	L	R	L	R	L	R	L	R
MEC	20	40	26	35	91	92	91	78
vHPC	-	-	-	-	79	80	83	75
vPreSub	-	-	-	-	87	77	89	80

Table 1: Percentage of MEC, ventral hippocampus, ventral presubiculum lesioned in the left and right hemispheres

very small amount (~12%) of spared tissue in the ventral MEC. Table 1 shows the lesion percentages for each animal.

Example sections from a control, small lesion and large lesion are shown in Figure 3a-c. All electrodes were located in dorsal hippocampus at approximately 2 mm posterior to bregma. Two example electrode positions are shown in Figure 3d.

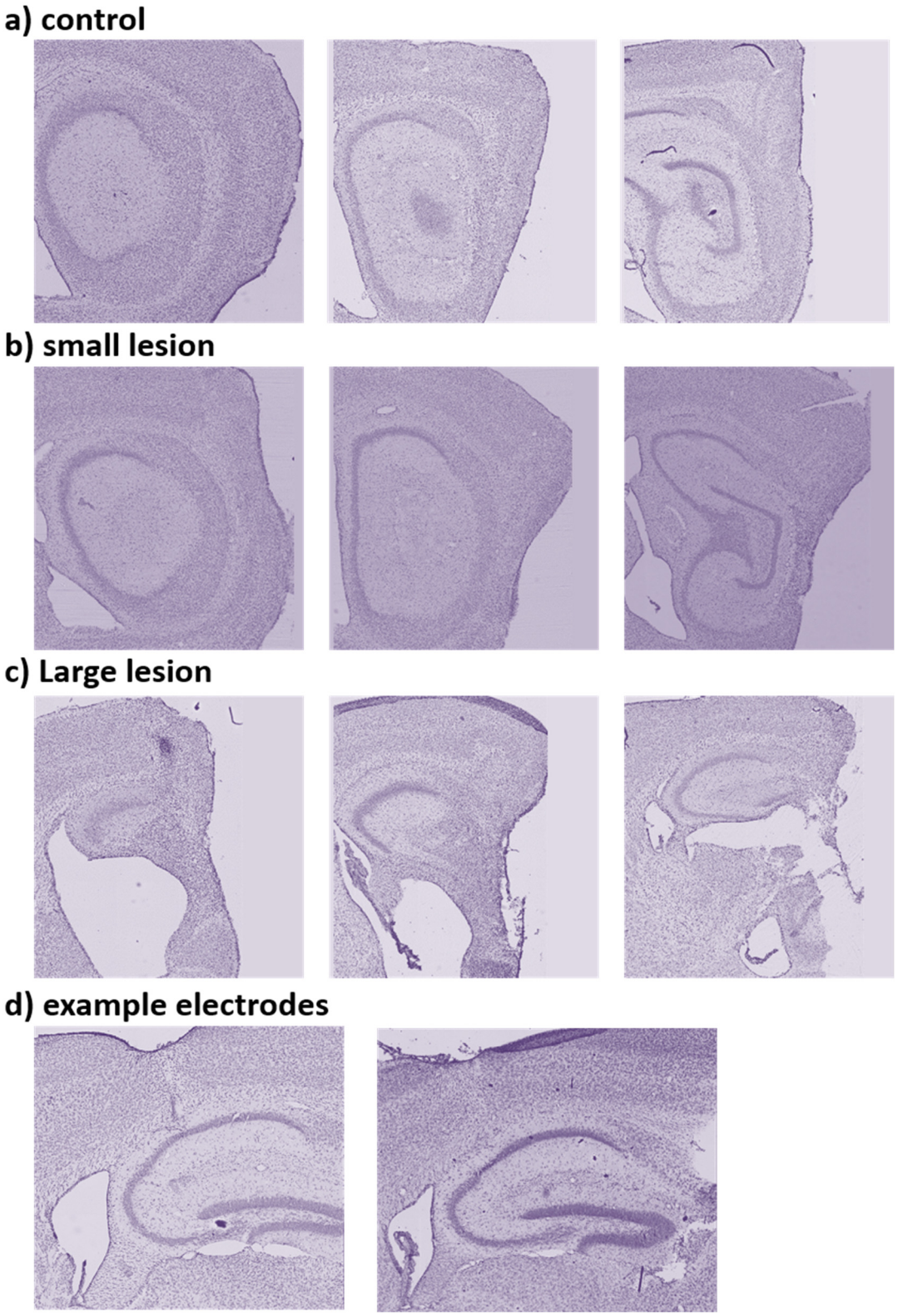


Figure 3: Histology: three example sections across the width of the MEC from a) a control animal, b) an animal with a small lesion and c) an animal with an extensive lesion. d) representative electrode positions from one of the controls and one of the lesions.

4.3.2 Identification of pyramidal neurons

Once cells which did not meet the criteria for a pyramidal cell were excluded, there remained 479 pyramidal cells in the sham group and 203 pyramidal cells in the lesion group. Because all the data using distal cues was collected first and the number of cells recorded tended to decrease across days, fewer cells were recorded during sessions with proximal cues than were recorded with distal cues. From the sham animals 322 pyramidal cells were recorded in the sessions with distal cues and 157 were recorded in the proximal cue sessions. In the lesion group 122 cells were recorded in the distal cue sessions and 81 cells in the proximal sessions. Table 2 below shows how many cells were recorded from each experimental animal. It was not possible to identify the same cells across day and so there is a possibility that some cells were recorded on multiple days.

	Sham Animals (4)				Lesion Animals (4)			
	1	2	3	4	1	2	3	4
Distal	14	28	91	189	21	13	49	39
Proximal	14	31	57	55	22	14	27	18

Table 2: Number of pyramidal cells recorded from each animal during distal and proximal sessions.

4.3.3 Place cell characteristics

The overall firing rate of each pyramidal cell across the whole recording session was calculated by dividing the total number of spikes by the total recording time. As can be seen in Figure 4a & b, the firing rates in both groups vary widely between 0.1 Hz (the threshold for inclusion), and 5 Hz (the maximum cut-off before cells were discarded as interneurons). The mean firing rate for both groups is just above 1Hz. The distribution is not normally distributed so a Mann-Whitney U test was used to test for a difference between the groups. There was no difference between the groups in the distal cue sessions $p=0.06$ or the proximal cue sessions $p=0.9$.

Since the overall firing rate can be affected by an animal's behaviour, particularly how much time the animal spends within a cell's place field, we also looked at the peak firing rate as this should be more representative of the infield firing rate of the cell.

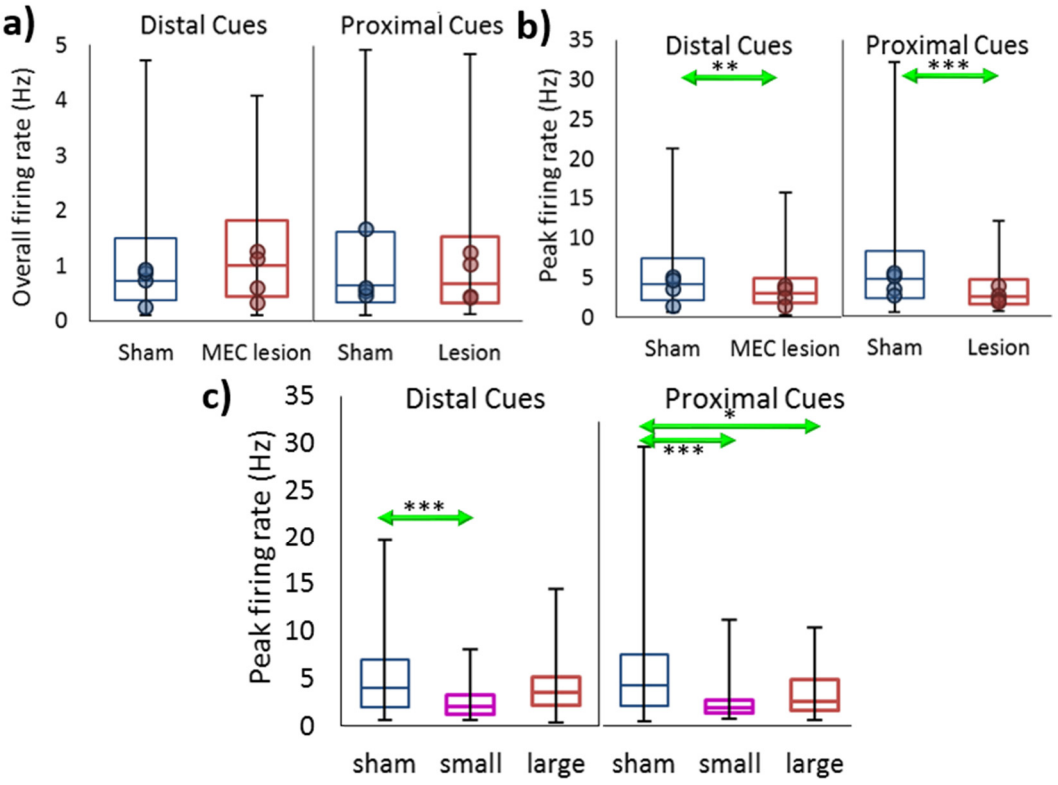


Figure 4: a) Box-whisker plot displaying overall firing rate during distal cue sessions and proximal cue sessions b) Box-whisker plot displaying peak firing rate during distal cue sessions and proximal cue sessions. Circles depict individual animal means. c) Box-whisker plot displaying peak firing rate with the lesion group divided into the small lesion group and the large lesion group. [Asterisks show statistical significance: * p<0.05, ** p<0.01, *** p<0.001]

The value of the pixel with the highest firing rate in the Gaussian-smoothed firing rate map was used as this measure of peak firing rate. There was a decrease in peak firing rate of around 30% from around 5 Hz in the sham group to between 3-4 Hz in the MEC lesion group (Figure 4c & d), which was statistically significant [Mann-Whitney U test; distal cue sessions: $p=0.003$, proximal cue sessions $p<0.001$. To check whether this difference was also seen across all four animals, the mean firing rates for each animal (plotted as circles on Figure 4) were compared across both session types with an RM-ANOVA, however there was no overall effect of group on peak firing rate when analysed by animal [$F_{(1,6)}=2.9$; $p>0.1$]. Closer inspection of the data revealed that one of the mice in the sham group who had contributed only a small number of pyramidal cells (28) had, a very low mean peak firing rate. Consequently, there is no

overall effect on peak firing rate when the groups are analysed by animal rather than grouping all cells together, but this is possibly due to the small sample size of cells from one subject. It has also been suggested that MEC lesions cause an increase in the proportion of low-firing cells (Peak-FR<1Hz) but do not affect the firing rates of cells which do have a peak-firing rate of >1 Hz (Hales *et al.* 2014). These statistical tests were therefore repeated on only those cells which had a peak firing rate of greater than 1 Hz. There was still a significant difference between the groups [Mann-Whitney distal: $p=0.01$, proximal: $p<0.001$] indicating that the change in peak firing rate is not driven by an increase in the amount of cells with a low firing rate but that MEC lesions reduce the peak firing rate of active cells.

To check whether the effect on peak firing rate was consistent across the different sizes of lesion, the data were also analysed separately for cells from the small and large lesion groups (Figure 4c). A Kruskal-Wallis test found a difference between groups [Distal: $p<0.005$, Proximal: $p<0.001$]. Interestingly, the effect on firing rate was stronger in the small lesion group. Post-hoc tests showed that for the distal sessions, only the small lesion group showed a significant reduction in peak firing rate compared to controls [$p<0.001$], the large group did not show a significant reduction in peak firing rate [$p=0.4$]. In the proximal sessions both small and large lesion groups showed a significant reduction compared to controls [Small: $p<0.001$, Large: $p<0.05$].

Since place cells from the MEC lesion group have a lower peak firing rate but do not have an overall lower firing rate, this suggests either that increased out of field firing may be responsible for the maintained overall firing rate, or alternatively that place field position may be more unstable over the length of the recording session, resulting in a lower firing rate in the centre of the place field because of field drift. This increased diffuseness and reduction in precision of place fields should show up as an increase in sparsity, which measures what percentage of the environment contains spikes, and a decrease in spatial information content, which measures how much information about an animal's location can be inferred from one spike.

Cells from the MEC lesion animals have more diffuse place fields than cells from the sham animals but place fields can still be seen. Figure 5a&b shows a cell from each group with sparsity and spatial information near the median values (more example rate maps are shown in Figures 6 & 10). Spikes from cells in the MEC lesion group tended to occur over a much larger proportion of the environment, with an average sparsity of 70% for cells in the MEC lesion group compared with 50% in the control group (Figure 5e) [Mann-Whitney U test; $p<0.001$ (for both distal and proximal sessions)]. The sparsity was also analysed for the small and large lesion group separately (figure 5f). There is a significant difference between the groups [Kruskal-Wallis $p<0.001$ for both distal and proximal sessions]. From the figure it appears that both groups show increased sparsity compared to the controls with larger lesions showing a greater increase. However, post-hoc tests revealed that only the large lesion group was significantly different from the sham group [Dunn-Bonferroni post hoc test. Large lesion group Distal: $p<0.001$, Proximal $p<0.005$, Small lesion group $p>0.1$].

The spatial information content was calculated using the equation developed by Skaggs *et al.* in 1993. As can be seen in Figure 5d & 5g, the spatial information of pyramidal neurons in the MEC lesion group is lower than in the control group. The average spatial information during a distal cue session for a place cell from the sham group is 0.63bits/spike while for lesion animals it is 42% lower at only 0.36bits/spike, and the same trend is seen in proximal sessions as well (Sham SI=0.72bits/spike Lesion SI=0.36bits/spike). This difference is statistically significant [Mann-Whitney U test; Distal: $p<0.001$, Proximal: $p<0.001$]. This indicates that each spike carries less information about the animal's current location suggesting that place cell activity is less precise. The spatial information was analysed for the small land large lesion groups separately (Figure 5h), again showing that the effect was stronger in the large lesion group. Again a Kruskal-Wallis test showed that there was a difference between groups $p<0.001$, and post-hoc tests showed that only the large lesion group was significantly different from the control group [Dunn-Bonferroni post hoc test for distal and proximal Large lesion group: $p<0.001$ Small lesion group $p>0.1$].

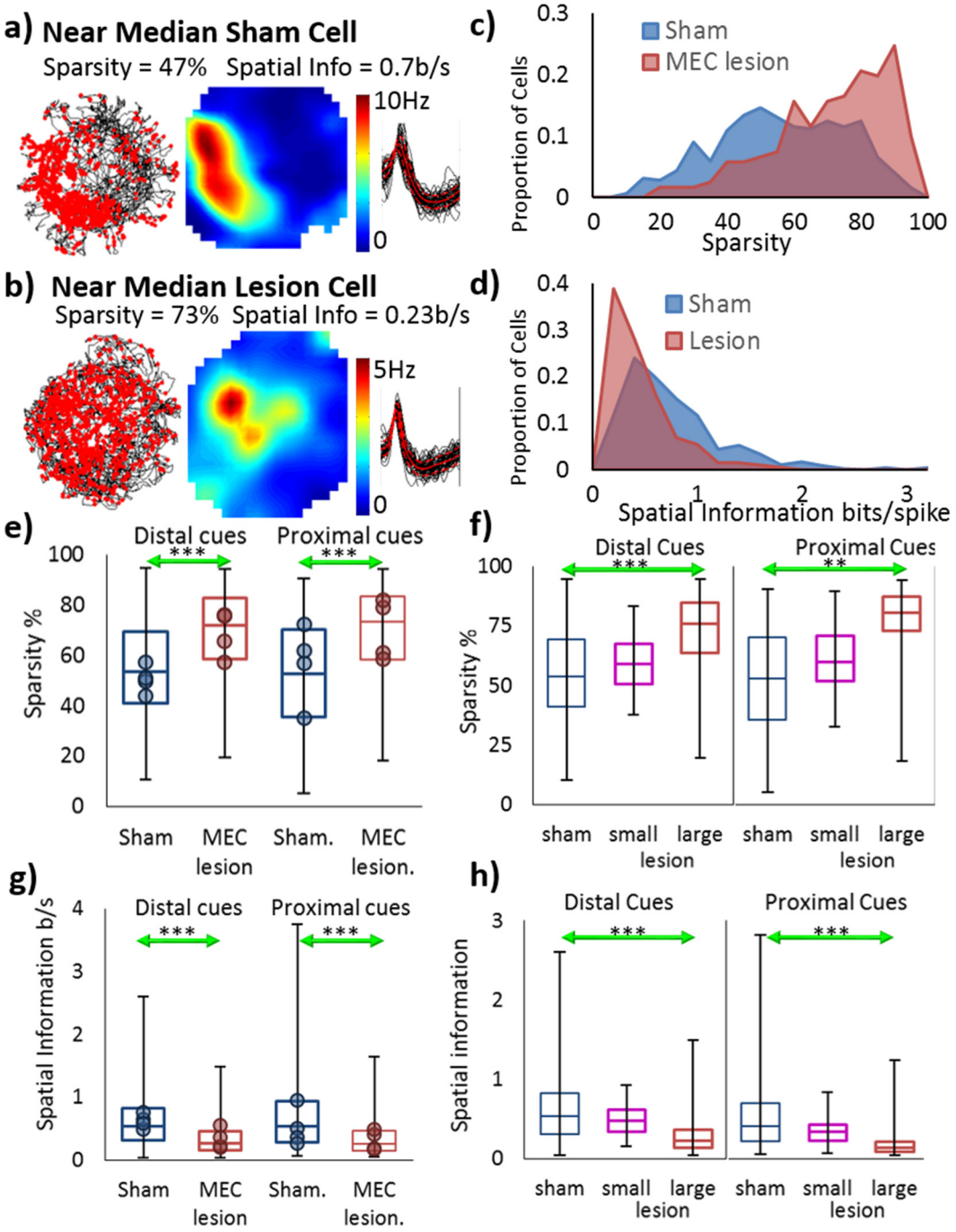


Figure 5: a) example cell from the sham group with a sparsity and spatial information near the median values b) example cell from the lesion group with a sparsity and spatial information near the median values c) distribution of sparsity for all cells d) distribution of spatial information for all cells e) box-whisker plot displaying sparsity during distal and proximal cue sessions (circles display individual animal means) f) sparsity with the lesion group divided into the small and large lesion groups g) box-whisker plot displaying spatial information during distal and proximal cue sessions (circles display individual animal medians) h) spatial information with the lesion group divided into small and large lesion groups [Asterisks show statistical significance: * p<0.05, ** p<0.01, *** p<0.001]

Both sparsity and spatial information were also analysed by animal. The mean spatial information and sparsity of cells in each animal was calculated and a RM-ANOVA was used to establish that there was a significant overall effect of group [ANOVA $F_{(1,6)}=6.3$; $p=0.045$] for sparsity and the same trend was apparent for spatial information although it did not reach significance [$F_{(1,6)}=5.7$; $p=0.05$]. These data show an overall decrease in spatial precision in place cells without an overall effect on firing rate of place cells.

4.3.4 Place field stability between distal cue sessions

194 pyramidal neurons from the shams and 52 pyramidal neurons from the lesion animals were active in both of the first standard sessions. An additional 54 cells in the sham group, and 24 cells in the lesion group were active in one session but not the other. This number includes cells which showed total remapping between sessions, but may also include cells which were lost from the recording due to drive instability. The proportion of cells active in only one of the two first standard sessions is shown in Table 3, but did not differ significantly between the groups [Chi-Square (1) = 3 $p>0.05$]. The first two columns in Figure 6 show the firing rate maps of example cells in standard sessions 1 and 2. The bottom two cells in the red box show cells which remapped between sessions 1 and 2, while the other example cells appear stable during the first two sessions. To quantify place field stability for each cell, the firing rate map of the second session was rotated in increments of 5° and the correlation with the first map was calculated at each angle to obtain the angle of best correlation. The angle of best correlation for all cells active in both sessions is displayed in a circular histogram (Figure 7a&b). A strong peak at 0° indicates that the majority of place cells in both the sham and lesion rats show fields in the same location in the two sessions.

	Number of Cells active in:		% active cells	
	Both	One	Both	One
Sham	194	54	78%	22%
Lesion	52	24	68%	32%

Table 3: The number of cells active in both or one of the two first standard sessions

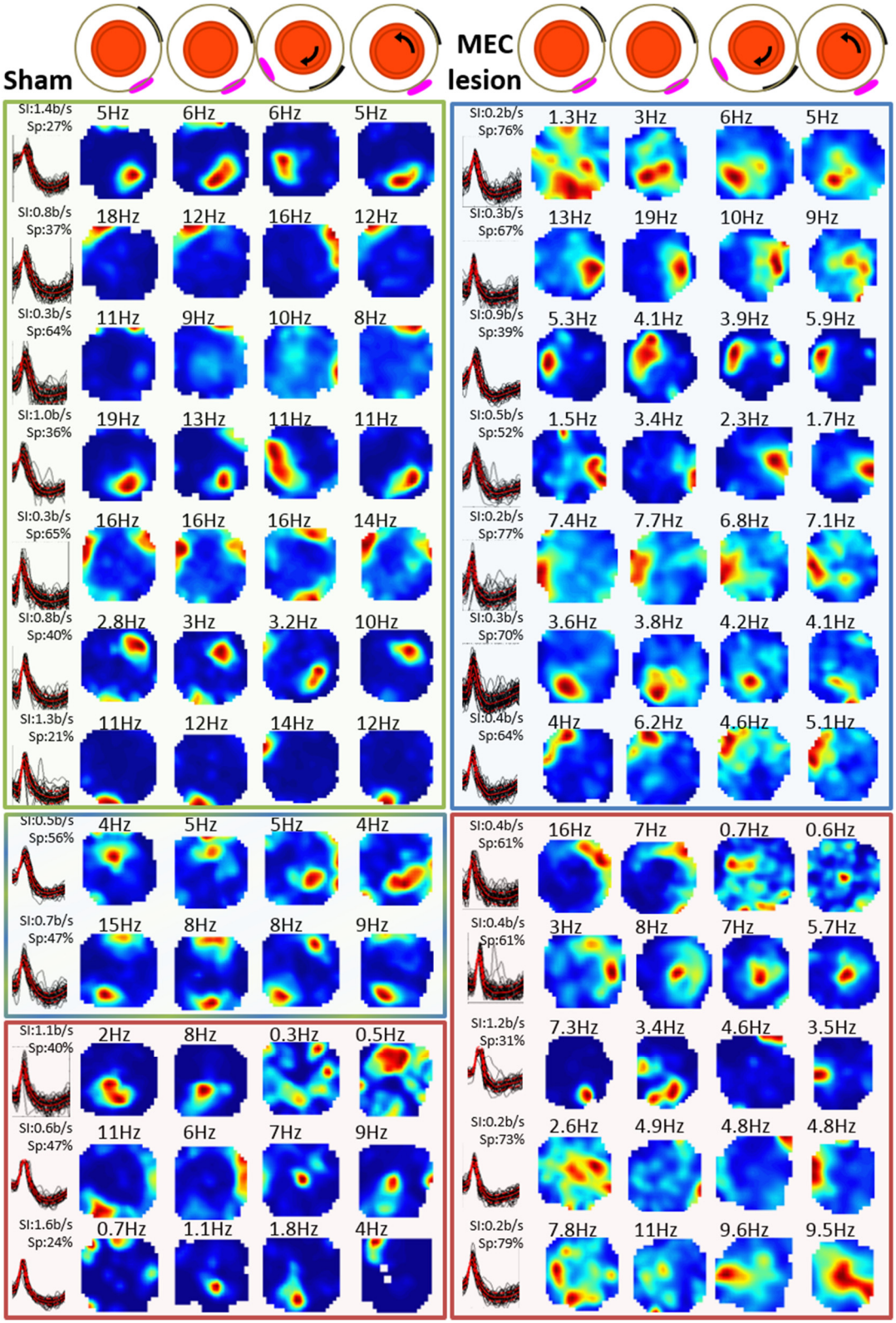


Figure 6: Example cells from the sham group (column 1) and the lesion group (column 2). Cells inside the green box showed rotation with the cues; cells inside the blue box showed stability across all sessions; cells inside the blue/green box showed incomplete rotation or a mix of rotation and stability; cells inside the red boxes showed remapping.

The proportion of cells which remained stable showing place fields in the same location in the two sessions was calculated compared with the proportion of cells which remapped showing fields in different locations (identified as rate map pairs with a max correlation <0.5 or an incorrect angle of best correlation) and is shown in Figure 7a & b. In the sham animals, 51% of cells remained stable, while in the lesion group, the proportion of stable cells was 52%. There was no significant difference in place cell stability between sessions when no cues are manipulated [Chi-Square(1)=0.03; p=0.9]. In addition, there was no difference in stability between the small lesion group and the large lesion group [Chi-Square(1)=2; p=0.2]. These results indicate that there is no difference in place field stability between sessions in which distal cues are available and no cues are manipulated.

4.3.5 Distal cue control of place fields

Again analysis was performed on pairs of sessions. Table 4 shows the number of cells which were active in each pair of rotation sessions, and the number which were only active in one of the two sessions. Again these numbers may reflect a combination of cells which showed global remapping and cells which were lost or gained during recorded because of electrode drift.

	Standard 2 - Rotation				Rotation – Standard 3			
	Number of Cells active in:		% cells active in:		Number of Cells active in:		% cells active in:	
	Both	One	Both	One	Both	One	Both	One
Sham	227	31	88%	12%	196	56	78%	22%
Lesion	46	17	63%	27%	58	15	79%	21%

Table 4: The number of cells active in both or one of each pair of rotation sessions

Comparing the behaviour of place fields which were active during both of the standard and rotation sessions revealed a profound difference between the groups. Figure 6 shows example cells from the sham group on the left and the MEC lesion group on the right, which either consistently follow cues across sessions (green box), consistently remain stable across sessions (blue box), rotate following the cues in one session-pair but remain stable with the room in another session-pair (blue/green box), or remap between sessions (red box). Far more cells in the sham group appear to show place fields which are anchored to distal cues and rotate in session 3. In contrast, many place fields from cells in the lesion group tend to remain stable ignoring the cue rotations.

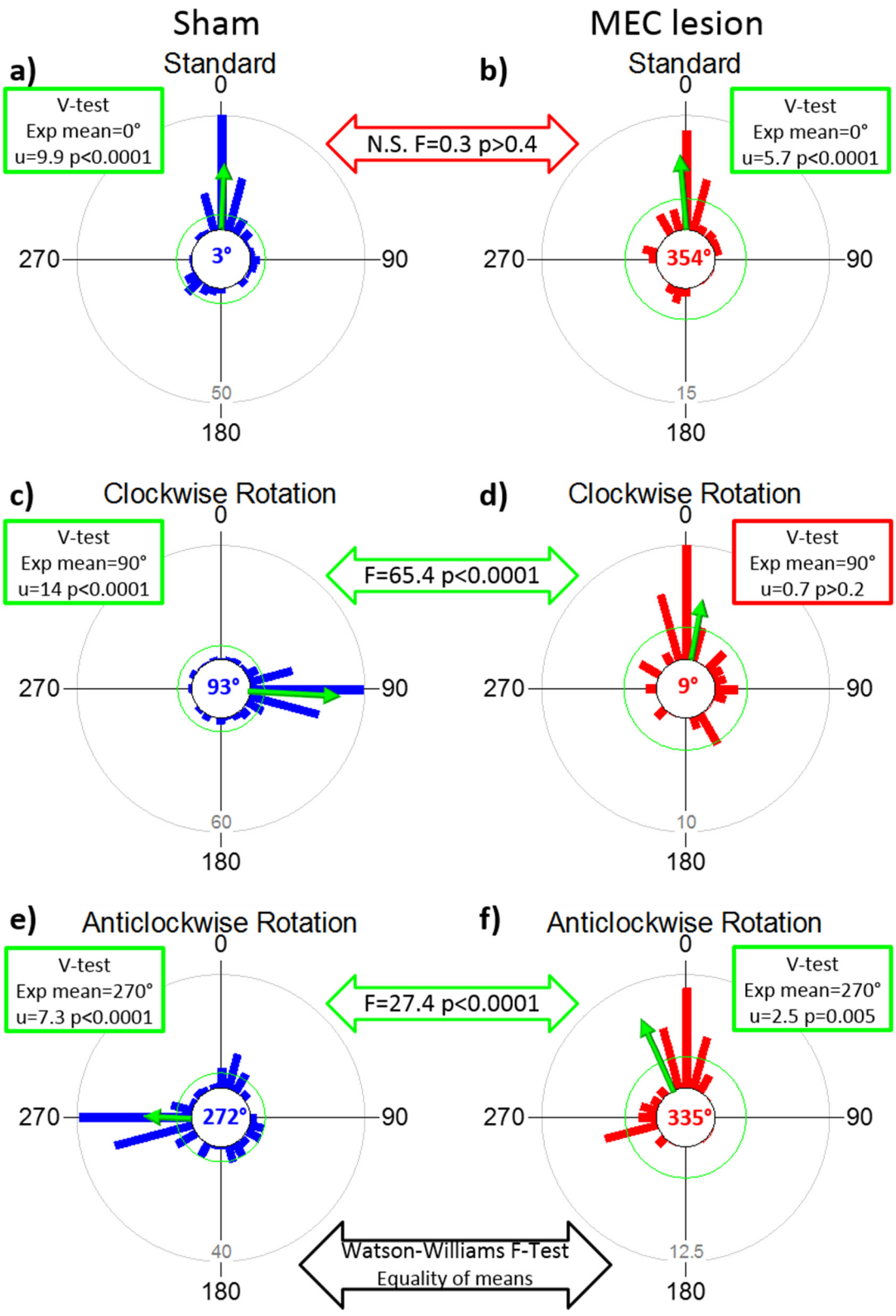


Figure 7: Angles of best correlation with distal cues (continued on next page)

Figure 7: (Previous page) Angles of best correlation with distal cues. Circular histograms displaying frequency of cells with each angle of best correlation (15° bins) for firing rate maps between standard sessions 1 and 2 (a & b), standard session 2 and rotation (c &d) and rotation and standard session 3 (e & f). Histograms a, c & e display data from all cells in the sham animals which were active in each pair of sessions, and histograms b, d & f display the same data from the MEC lesion animals. The number in the centre of each plot displays the mean angle (μ), which is also displayed as a green arrow. The length of the arrow (r) is a measure of how tightly the distribution is clustered around this mean. The green circle displays the Rayleigh's critical value for the data. If the mean vector arrow passes outside the circle then the distribution shows a significant deviation from uniformity. Values inside boxes display the results of a V-test which tests deviation from uniformity, towards the mean expected if place fields followed the controlled cues. Values inside double arrows display the results of a Watson-Williams F-test for equality of means between the groups for each pair of sessions. a & b) The angles of best correlation of both the sham and MEC lesion groups are tightly clustered around 0° as would be expected since no cues change between standard sessions. There is no difference between the groups. c & d) The angles of best correlation for the sham group are tightly clustered around 90° as would be expected if place fields followed the controlled distal cues. Conversely the angles of best correlation for the MEC lesion group are clustered around 0° and show increased variability, suggesting that place fields did not follow the rotated cues. There is a significant difference between the two groups. e & f) The angles of best correlation for the sham group are tightly clustered around 270° as would be expected if place fields followed the controlled distal cues as they were rotated back around the room. The angles of best correlation for the MEC lesion group show increased variability, with a peak at 0° and a smaller peak at 255° suggesting that most place fields did not follow the rotated cues. There is a significant difference between the two groups.

Figure 8: (Next page) Responses of place cells to distal cue rotations. Pie charts displaying the number of cells which remained stable (blue), rotated (green), or remapped (red) between each pair of distal cue sessions. Numbers inside pie chart display number of cells. a & b) Proportions of cells which remapped or stayed stable between standard sessions 1 & 2. c & d) Proportions of cells which rotated, remapped, or stayed stable between standard session 2 and the rotation session e & f) Proportions of cells which rotated, remapped or stayed stable between the rotation session and standard session 3.

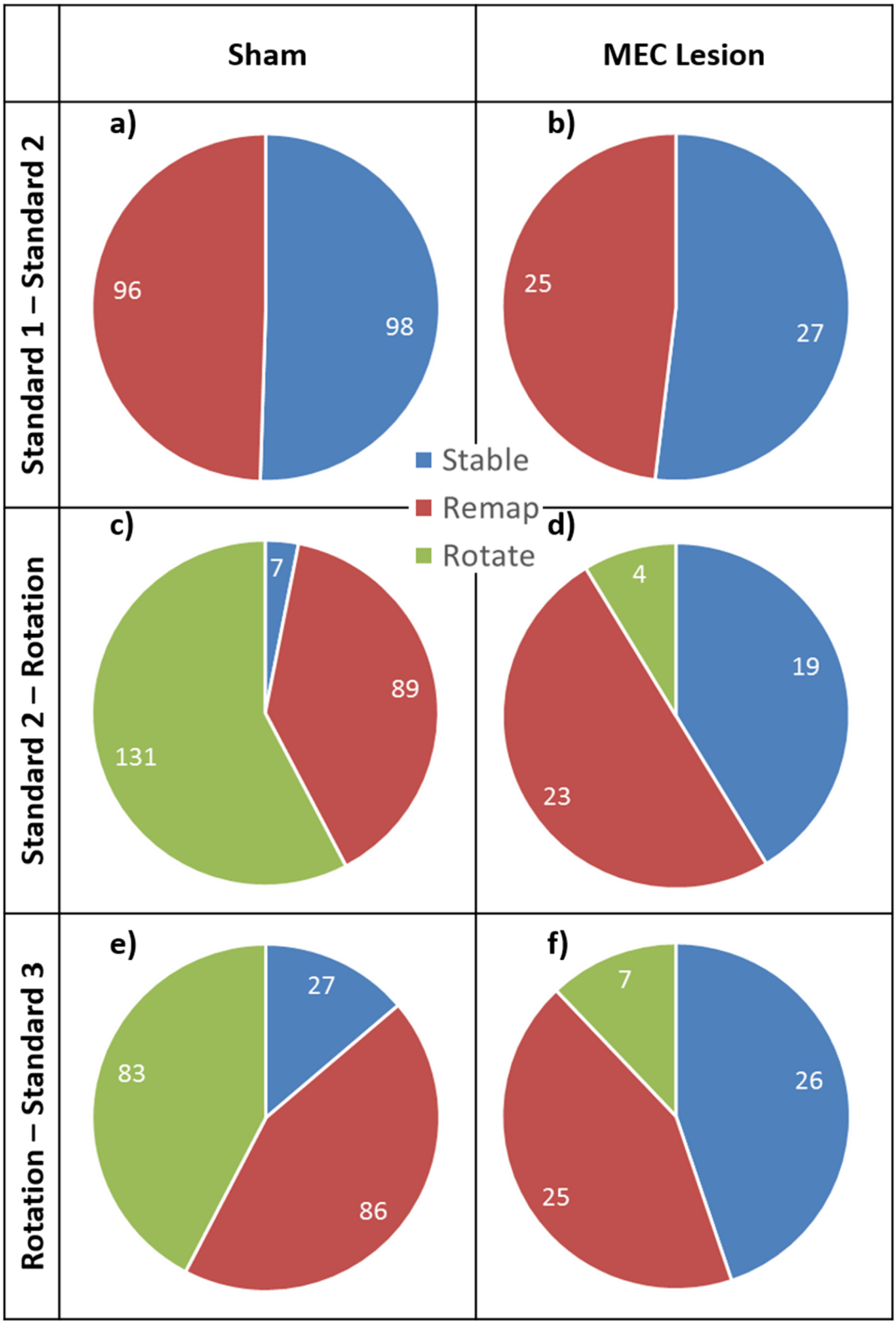


Figure 8: Categorical analysis of place field responses to distal cues

Plotting the angle of best correlation for each cell between pairs of sessions confirms this. Between the standard 2 session and the rotation session, the control group has a strong peak at 90° (Figure 7c), the lesion group shows a slightly weaker peak at 0° but no peak at 90° (Figure 7d). The circular distributions were compared using a Watson-Williams F-test and found to be significantly different [$F=65.2$; $p<0.0001$]. By categorising cells as rotating, stable or remapping it was observed that whereas 58% of cells rotated with the cues in the control group (Figure 8c &d), only 9% of place cells rotated with the cues in the MEC group. In contrast, 41% of cells in the lesion group remained stable, while only 3% remained stable in the sham group. The proportion of cells which remapped, rotated or remained stable were compared with a Chi-Square test and were found to be significantly different [Chi square(2)=78; $p<0.001$], suggesting that place cells from animals with MEC lesions do not rotate in response to rotations of distal cues, but instead remap or remain stable.

These comparisons were also performed between the rotation session and standard session 3. Again most cells in the sham group showed angles of rotation similar to the rotation of the cues (270°), while very few cells from the MEC lesion group showed this rotation (Figure 7e & f). The circular distributions were compared using a Watson-Williams F-test and found to be significantly different [$F=27.4$; $p<0.0001$]. Categorising cells as stable, rotating or remapping again showed that a much higher proportion of place cells (42%) in the sham group followed the distal cues than followed the cues in the lesion group (12%), and more lesion cells remained stable (45%) compared with shams (14%) (Figure 8e & f). This difference was also significant [Chi-Square(2)=32; $p<0.001$].

From Figure 8d & f, it can be seen that a small proportion of cells in the lesion group appeared to rotate following the distal cues. The proportion of cells which rotated clockwise between the standard sessions was used to provide an indication of the proportion of cells which might appear to rotate with the cues but actually be the result of chance remapping to a field approximately 90° around the maze. The proportion of cells showing rotation in the lesion group was not significantly different in the cue-rotation session-pairs compared with the proportion that showed rotation in the

standard-standard session pair. [Chi-Square=2.9; p=0.24].This indicates that any remaining cue-rotation apparent in this group may simply be the result of random remapping rather than cue-control of place fields. Conversely, the sham group had a highly significant difference in proportion of rotating cells in the rotation session pairs compared with the standard pairs [Chi-Square=132; p<0.001]. This result is consistent with the absence of a peak at 90° in the circular distribution for the clockwise cue-rotation (Figure 7d), and provides further evidence that place cells in MEC lesion animals are not anchored to distal cues.

4.3.6 Spatial Information control

To ensure that the decrease in distal cue rotation was not merely an effect of the lower spatial precision of place cells in the MEC lesion group, the rotation analysis was repeated using only the cells which passed a spatial information threshold of 0.5bits/spike. This is the usual cut off used to select for place cells in other papers but was not applied to this data previously because one of the aims of the study was to measure the effect of MEC lesions on spatial information. In addition the data were also analysed with a cut off of 0.3bits/spike in order to include more cells but still reduce the effect of cells with very low spatial precision. This cut off is slightly lower than the usual cut off but visual examination of example cells with a spatial information of 0.3bits/spike confirmed that place fields were clearly visible with this level of spatial information (See Figure 6 : Column 2 (MEC lesion) examples 2, 6 & 7). The data from each band of spatial information threshold is displayed in Figure 9. The inner circle shows the data from only the cells with highest spatial information (>0.5bits/spike), and the middle band shows the data from cells which passed the 0.3bits/spike threshold. The same distribution is seen in each band as tested with a Chi-Square test (or Fisher's exact test in cases with fewer than 5 cells in a category) (p>0.1 for all conditions). This suggests that the reduced control of place fields by distal cues in the MEC lesioned rats is not due to the reduction in spatial information.

Figure 9: (Next page) Concentric pie charts displaying the number of cells which remained stable (blue), rotated (green), or remapped (red) between each pair of distal cue sessions. The inner circle displays proportions from only cells which passed the spatial information threshold of >0.5bits/spike, the middle circle displays proportions from cells which passed the spatial information threshold of >0.3bits/spike and the outer circle displays proportions from all cells. Numbers inside pie chart display number of cells.

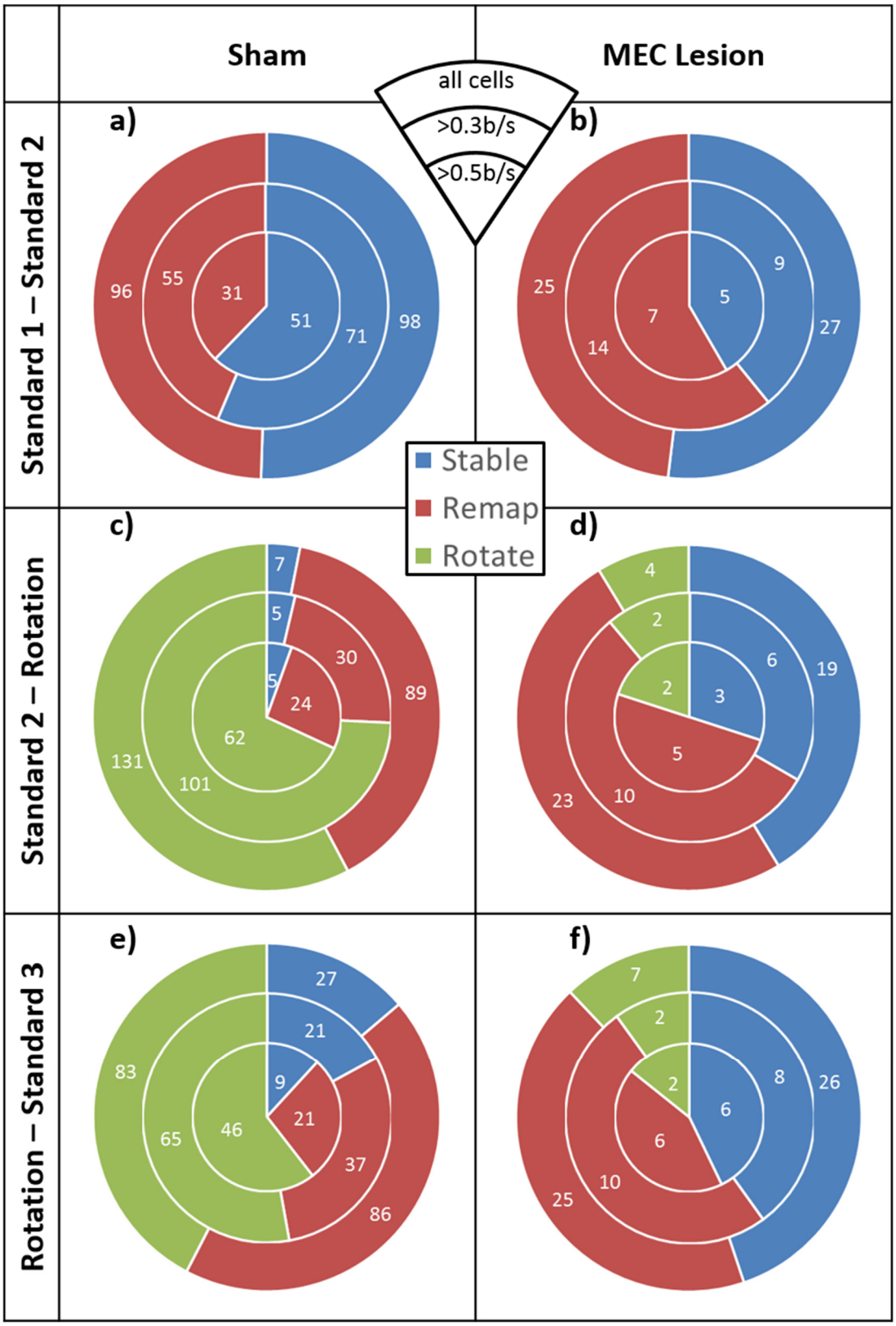


Figure 9: (Continued)a & b) Proportions of cells which remapped or stayed stable between standard sessions 1 & 2. c & d) Proportions of cells which rotated, remapped, or stayed stable between standard session 2 and the rotation session e & f) Proportions of cells which rotated, remapped or stayed stable between the rotation session and standard session 3.

4.3.7 Place field stability with proximal cues

105 pyramidal neurons from the shams and 52 pyramidal neurons from the lesion animals were active in both sessions. In addition, 16 cells from the sham group and 16 cells from the lesion group were active in only one of the two sessions. This number includes cells which showed total remapping between sessions, but may also include cells which were lost from the recording due to drive instability. The proportion of cells active in only one of the two first standard sessions is shown in Table 5, but did not differ significantly between the groups [Chi-Square (1)=3; p>0.05].

	Number of Cells active in:		% active cells	
	Both	One	Both	One
Sham	105	16	87%	13%
Lesion	52	16	76%	24%

Table 5: The number of cells active in both or one of the two first standard sessions

Example cells which were active in both of the first two standard sessions are displayed in the first two column of rate maps in Figure 11. It can be seen that place fields in both groups showed a level of stability between the first two sessions. The place field stability between the two standard sessions with proximal cues was calculated in the same way as for the distal cue standard sessions. The angle of best correlation for all cells is displayed in a circular histogram (Figure 10a & b). The strong peak at 0° seen in both sham and lesion groups indicates that the majority of place cells show fields in the same location in the two sessions. The proportion of cells which remained stable showing place fields in the same location in the two sessions was calculated compared with the proportion of cells which remapped and is shown in Figure 11a & b. In the sham animals, 57% of cells remained stable, while in the lesion group, the proportion of stable cells was only 33%. This difference was significant [Chi-Square(1)=8.3; p=0.004], indicating that place fields from the lesion group are less stable between sessions. In the lesion group there was a slight decrease in stability the standard proximal cue sessions compared with between the standard distal cue sessions, however this was not significant [Chi-square (1)=4; p=0.06]. Comparing the stability of place fields in the standard distal and standard proximal session pairs, there was no difference in stability in the sham group [Chi-square p>0.1], but the lesion group

showed a significant decrease in stability in the proximal session pairs compared to the distal sessions [Chi-square (1) = 3.9; p=0.047], indicating that in the lesion group, proximal cues increased the instability of place cells.

4.3.8 Cue rotation with proximal Cues

Again analysis was performed on pairs of sessions. Table 6 shows the number of cells that were active in each pair of rotation sessions, and the number that were only active in one of the two sessions. Again these numbers may reflect a combination of cells

	Standard 2 - Rotation				Rotation – Standard 3			
	Number of Cells active in:		% cells active in:		Number of Cells active in:		% cells active in:	
	Both	One	Both	One	Both	One	Both	One
Sham	110	24	82%	18%	103	23	82%	18%
Lesion	50	7	88%	12%	56	11	84%	16%

Table 6: The number of cells active in both or one of each pair of rotation sessions

which showed global remapping and cells which were lost or gained during recorded because of electrode drift. There is no significant difference between groups in the proportion of cells active in only one session [Chi-square p>0.05].

Comparing the firing rate maps for cells active in both the standard and rotation sessions revealed a surprising result. In contrast to sessions with distal cues, in which place fields from the lesion group did not rotate, with proximal cues, many place fields from the MEC lesion group did rotate while the sham place fields remained stable. Figure 10 shows example cells from the sham group on the left and the MEC lesion group on the right, which either consistently follow proximal cues across sessions (green box), consistently remain stable across sessions (blue box), rotate following the proximal cues in one session-pair but remain stable with the room in another session-pair (blue/green box), or remap completely between sessions (red box). Although the place fields are larger and less well defined in the lesion group, it is still obvious that they are more likely to rotate following the cues than cells from the control group, which tend to be unstable or remain stable ignoring the rotating cues.

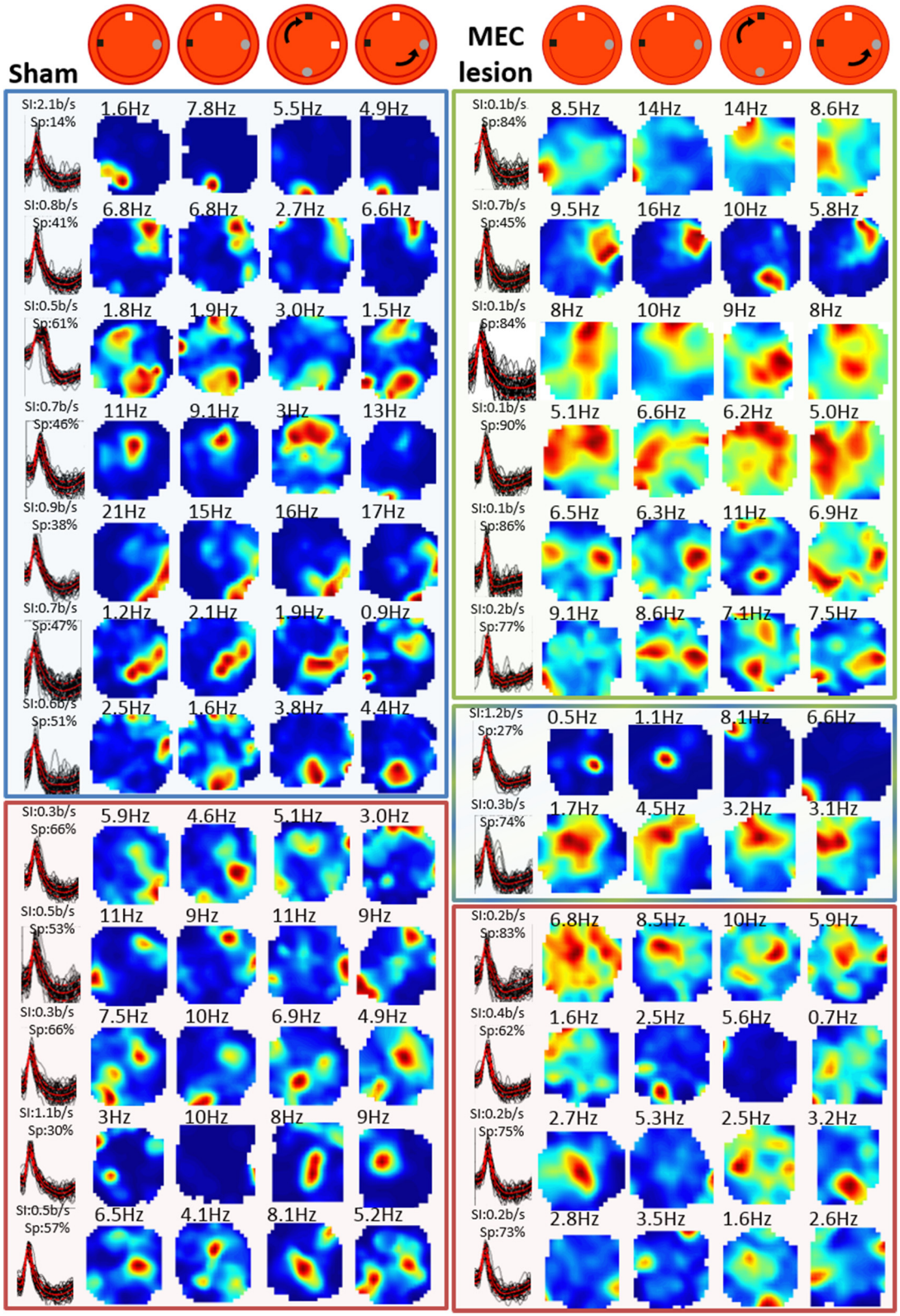


Figure 10: Example cells from the sham group (column 1) and the lesion group (column 2). Cells inside the blue box showed stability across all sessions; cells inside the green box showed rotation with the cues; cells inside the blue/green box showed incomplete rotation or a mix of rotation and stability; cells inside the red boxes showed remapping.

By rotating the firing rate maps and plotting the distribution of angles of best correlation it can be seen that for the sham group the distribution of the angles of best correlation showed a peak at 0° (Figure 11c), although a smaller proportion of cells did this. However in the lesion group most of the cells showed best correlation at angles near 90° (Figure 11d) suggesting that the place fields rotated following the distal cues. The circular distributions were compared using a Watson-Williams F-test and found to be significantly different [$F=31.5$; $p<0.0001$]. By categorising cells as rotating, stable or remapping it was observed that whereas only 5% of cells rotated with the cues in the control group (Figure 12c), 36% of place cells rotated with the cues in the MEC group (Figure 12d). In contrast, 33% of cells in the sham group remained stable, while only 5% remained stable in the MEC lesion group. The proportion of cells which remapped, rotated or remained stable were compared with a Chi-Square test and were found to be significantly different [Chi square(2)=20.9; $p<0.001$], suggesting that place fields in the lesion group are more likely to rotate following proximal cues, and less likely to remain stable with the room than place fields in control animals.

These comparisons were also performed between the rotation session and standard session 3. Again, the largest peak in the distribution of angles of rotation in the sham group was near to 0° (Figure 11e), although there was a small peak around 270°. However, the majority of cells in the MEC lesion group rotated, following the proximal cues (Figure 11f). The circular distributions were compared using a Watson-Williams F-test but were not significantly different [$F=3.0$; $p=0.09$]. Categorising cells responses it was found that a much higher proportion of place cells (36%) in the MEC lesion group followed the proximal cues than followed the cues in the sham group (18%), and fewer lesion cells remained stable (5% compared with shams (27%) (Figure 12e & f). Again this difference was significant [Chi-Square(2)=13; $p=0.0012$].

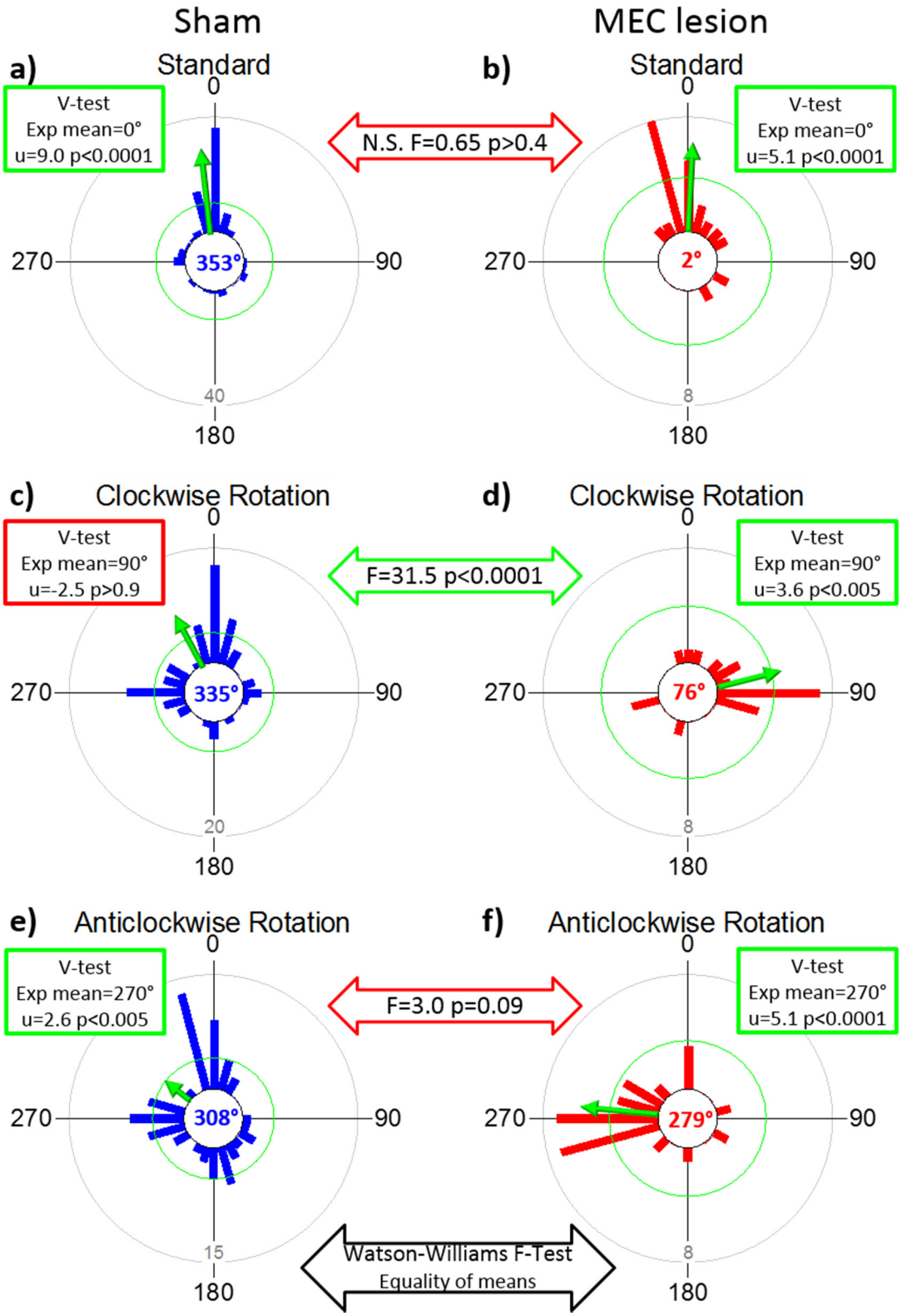


Figure 11: Angles of best correlation with proximal cues (legend continued on next page)

Figure 11: (Previous page) Angles of best correlation with proximal cues. Circular histograms displaying frequency of cells with each angle of best correlation (15° bins) for firing rate maps between standard sessions 1 and 2 (a & b), standard session 2 and rotation (c &d) and rotation and standard session 3 (e & f). Histograms a, c & e display data from all cells in the sham animals which were active in each pair of sessions, and histograms b, d & f display the same data from the MEC lesion animals. The number in the centre of each plot displays the mean angle (μ), which is also displayed as a green arrow. The length of the arrow (r) is a measure of how tightly the distribution is clustered around this mean. The green circle displays the Rayleigh's critical value for the data. If the mean vector arrow passes outside the circle then the distribution shows a significant deviation from uniformity. Values inside boxes display the results of a V-test which tests deviation from uniformity, towards the mean expected if place fields followed the controlled cues. Values inside double arrows display the results of a Watson-Williams F-test for equality of means between the groups for each pair of sessions. a & b) The angles of best correlation of both the sham and MEC lesion groups are tightly clustered around 0° as would be expected since no cues change between standard sessions. There is no difference between the groups. c & d) The angles of best correlation for the sham group have a peak at 0° but show a lot of variability, suggesting that the cells do not respond coherently to the cue-rotation. Conversely the angles of best correlation for the MEC lesion group are tightly clustered around 90° as would be expected if place fields suggesting that the place fields were anchored to the rotating proximal cues. There is a significant difference between the two groups. e & f) There is a lot of variability in angles of best correlation for the sham group, although the distribution is significantly different from a uniform distribution. The angles of best correlation for the MEC group show strong clustering around 270° as would be expected if place fields followed the proximal cue rotation. There is no significant difference between the groups although because of the low concentration of the sham distribution, this result may be unreliable.

Figure 12: (Next page) Responses of place cells to proximal cue rotation. Pie charts displaying the number of cells which remained stable (blue), rotated (green), or remapped (red) between each pair of proximal cue sessions. Numbers inside pie chart display number of cells. a & b) Proportions of cells which remapped or stayed stable between standard sessions 1 & 2. c & d) Proportions of cells which rotated, remapped, or stayed stable between standard session 2 and the rotation session e & f) Proportions of cells which rotated, remapped or stayed stable between the rotation session and

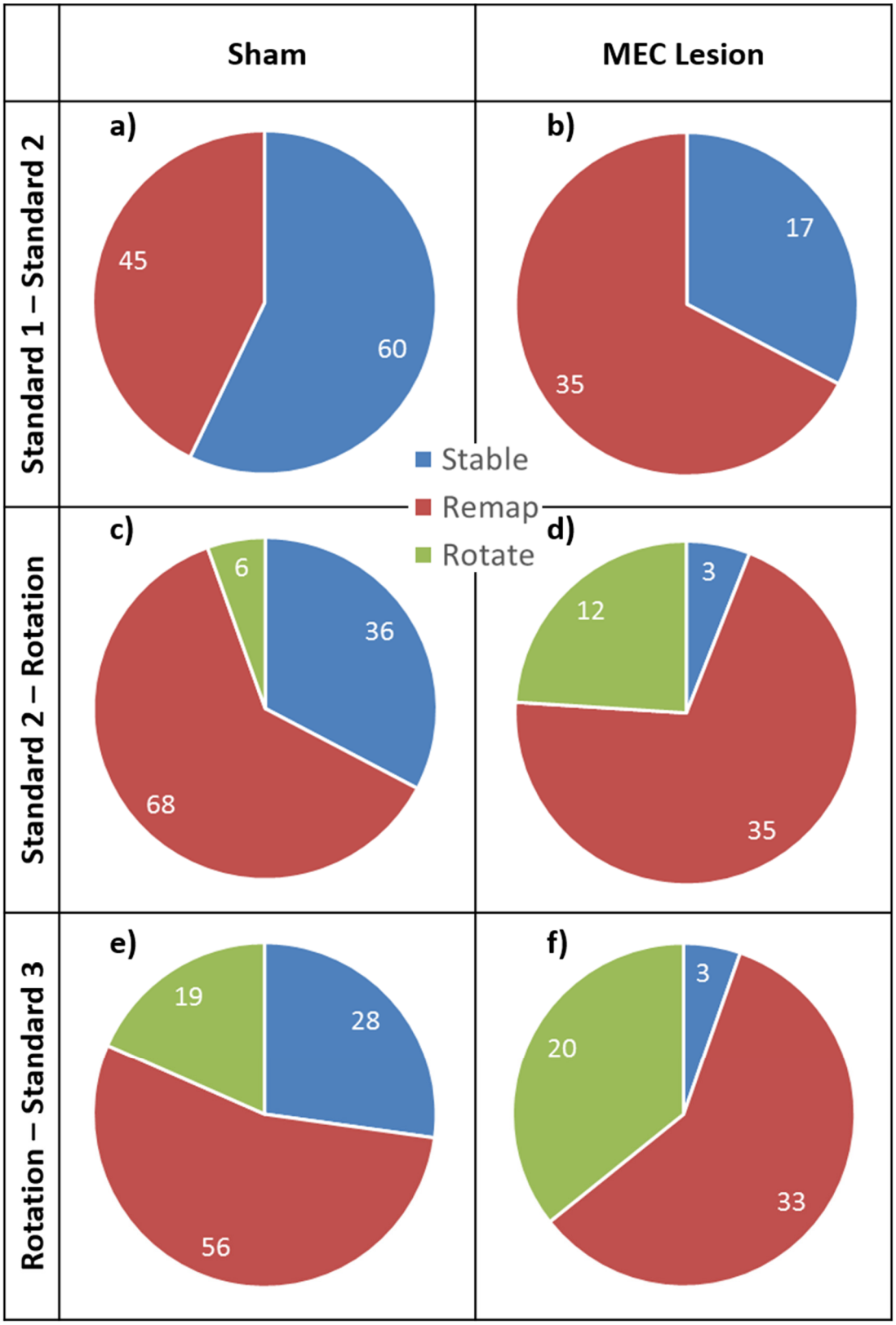


Figure 12: Categorical analysis of place field responses to proximal cues

4.3.9 Cluster quality

To check whether any of the effects reported here could be the result of poor cluster isolation, the data were reanalysed using only the better isolated clusters. Isolation distance and L_{ratio} were used to create bands of increasing cluster quality or isolation from noise and other clusters. Cells with higher cluster quality should be less likely to be contaminated by noise, and are also less likely to be affected by spikes from other neurons with similar waveform signatures. Figure 13 illustrates the position of the different cut-offs used to create each cluster quality band.

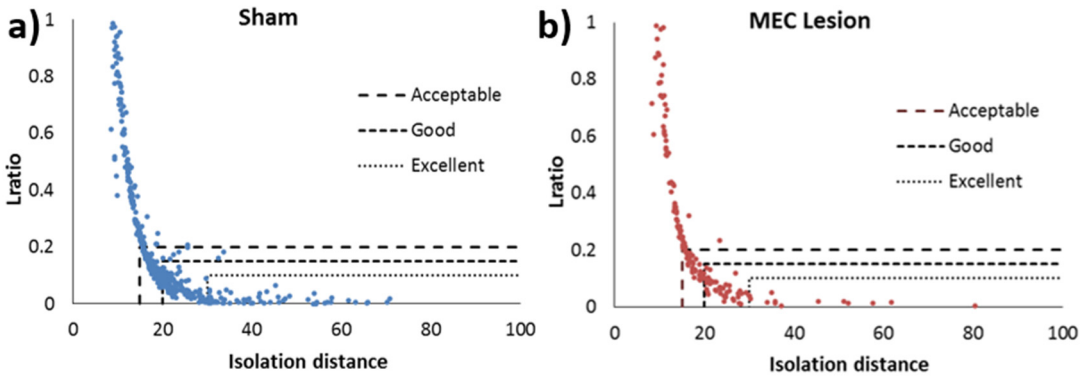


Figure 13: Plot of the isolation distance against Lratio for every cell from a) the sham group and b) the lesion group. Wide dashed line displays the cut off for acceptable levels of isolation distance and Lratio; narrow dashed line displays the cut off for good levels of isolation distance and Lratio; dotted line displays the cut off for excellent levels of isolation distance and Lratio.

Table 7 below shows the numbers of cells in each band of cluster quality after applying these thresholds.

	All clusters	Acceptable	Good	Excellent
	Thresholds →	ID > 15 L_{ratio} < 0.2	ID > 20 L_{ratio} < 0.15	ID > 30 L_{ratio} < 0.1
Sham	479 (211)	268 (97)	171 (108)	63
Lesion	203 (114)	89 (34)	55 (41)	14

Table 7: The number of cells which passed each set of thresholds (numbers outside of brackets), and number of cells within each band – passing the thresholds for that band but not passing the thresholds for the next band (numbers inside brackets).

Each measure of place cell activity and field precision was compared at each band of cluster quality. Both of the two measures of spatial precision; spatial information content and sparsity remained significantly different between the two groups at every level of the analysis. This indicates that the difference observed between the groups is not due to contamination from noise spikes. However, the difference in maximum firing rate was no longer significant at higher bands of cluster quality [Mann-Whitney U-test $p>0.05$]. This indicates that the differences in firing rate observed may be due to the effect of noise spikes contaminating the clusters. Table 8 below shows the averages for each band of cluster quality.

Median Values		All clusters	Poor	Acceptable	Good	Excellent
Firing Rate	Sham	0.70	0.50	0.97	0.92	1.24
	Lesion	0.88	0.57	1.1	1.6	2.0
Peak FiringRate	Sham	4.3	2.5	5.1	5.7	7.3
	Lesion	2.7	2.0	3.4	4.6	8.2
Spatial Info	Sham	0.54	0.46	0.62	0.60	0.66
	Lesion	0.27	0.29	0.23	0.24	0.19
Sparsity	Sham	53	58	49	51	47
	Lesion	72	70	75	76	78

Table 8: The median values for overall firing rate (Hz), peak firing rate (Hz), spatial information content (bits/spike) and Sparsity (%) for cells within each band of cluster quality (within a band but not in the higher bands).

The angle of best correlation was also analysed for each band of cluster quality and is displayed in Figure 13. It can be seen that the effects on cue rotation previously described are present at the highest levels of cluster quality suggesting that the effects are not driven by the poor quality clusters. The only notable difference between the different cluster quality bands, is that for the distal cue sessions, there is more variability in angle of best correlation during the cue rotation session pairs for the higher quality bands. However, the higher cluster bands do not show an increased tendency to follow the rotating cues, suggesting that the overall result that place cells from MEC lesion animals are not anchored to distal cues is unaffected by the quality of the clusters.

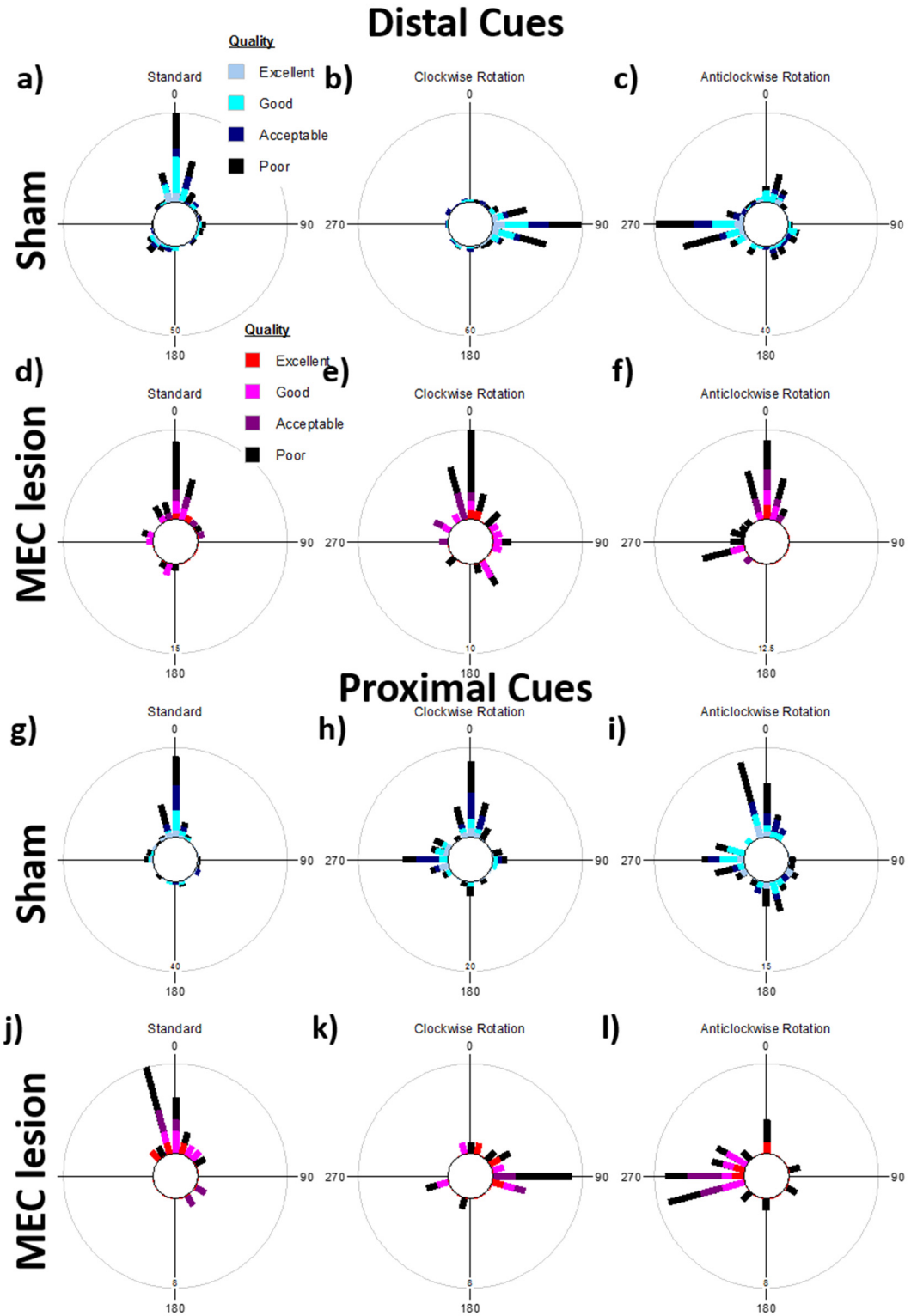


Figure 14: Circular histograms displaying frequency of cells with each angle of best correlation (15° bins) for firing rate maps between standard sessions 1 and 2 (a, d, g & j), standard session 2 and rotation (b, e, h & k) and rotation and standard session 3 (c, f, i & l). Histograms a-f) display data from all cells in the sham animals which were active in each pair of sessions, and histograms g-l) display the same data from the MEC lesion animals. The different colours represent the data from each band of cluster quality with lighter colours representing the highest band through to black which represents cells with poor cluster quality. The same trends can be seen in the central coloured bands, implying that the effects are not driven by cells with poor cluster quality.

This analysis was confirmed statistically using Rayleigh Test for uniformity at each cluster quality band, and a Watson-Williams Test for equality of means between quality bands for each session pair. These tests confirm that the mean angle of best correlation does not differ between quality bands for the shams during any of the session pairs (Watson-Williams test: $p>0.1$ for all combinations), nor for the lesions during most of the distal and proximal sessions, although there was a significant difference between the best quality clusters and the poor quality cluster for the clockwise distal cue rotation session pair (Watson-Williams test $p<0.05$) (Watson-Williams test $p>0.05$ for all other combinations). However, neither a Rayleigh Test for uniformity nor a V-test with an expected mean of 90° found any evidence that the distribution of angles of best correlation for lesion cells were not uniform during the clockwise distal cue rotation (Rayleigh Test $Z=1.98$; $p>0.1$; V-Test $u=1.4$; $p>0.05$). This implies that the difference in distribution of angles of best correlation between the cluster quality bands is due to a uniform distribution at the higher cluster quality bands suggesting place fields from well isolated cells in the lesion animals showed no coherent response to distal cues, while place fields from less well isolated cells appeared to remain stable relative to the room rather than following distal cues. Therefore the only observed difference between the cluster quality bands does not contradict the result that place cells from MEC lesion animals are not anchored to distal cues.

4.3.10 **Effect of lesion size on cue rotation**

The effects described here are dependent upon the size of the lesion. During distal cue sessions, animals with small lesions do show weak cue rotation, unlike animals with large lesions (Figure 15). However, place field location is slightly less tightly controlled by the distal cues than in the control animals. Although the mean angle of rotation is close to the cue rotation angles of +/-90° there is a much lower concentration about that mean with a non-significant Rayleigh's test for uniformity $p>0.05$ indicating that there is not a significant deviation from a uniform distribution in the small lesion group. In addition, although a similar proportion of place cells were categorised as rotating in the small lesion group as in the sham group, fewer place fields showed consistent rotation across more than one session pair than in the sham group (Figure 15b) [Chi-square(1)=5; $p<0.05$]. Place fields in the large lesion group showed no rotation with the cues, but 33% of cells showed stability across all three sessions. In

contrast no place cells in the small lesion group remained stable between sessions. These results indicate that the effect of small MEC lesions upon distal cue use is much less pronounced, although there is a reduction in the precision and stability with which place fields follow distal cues.

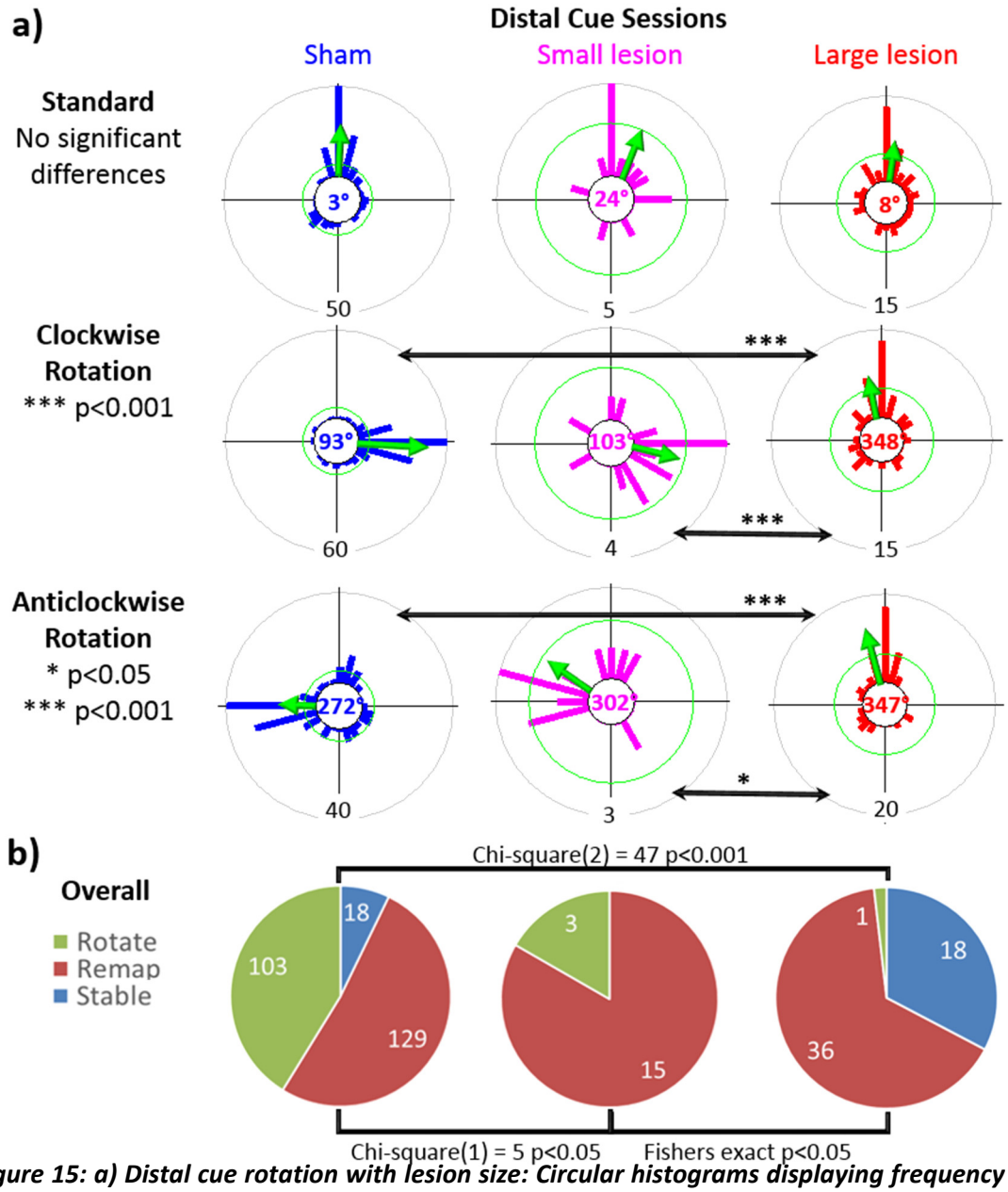


Figure 15: a) Distal cue rotation with lesion size: Circular histograms displaying frequency of cells with each angle of best correlation (15° bins) for firing rate maps between standard sessions 1 and 2 (row 1), standard session 2 and rotation (row 2) and rotation and standard session 3 (row 3). The number in the centre of each plot displays the mean angle (μ), which is also displayed as a green arrow. The length of the arrow (r) is a measure of how tightly the distribution is clustered around this mean. The green circle displays the Rayleigh's critical value for the data. b) Pie chart showing the overall behaviour of the cells in each experimental group categorised as either showing remapping, stability relative to the room, or stability relative to the cues across 3 sessions.

During proximal cue sessions, animals with small lesions resemble animals with large lesions more closely, with a mean angle close to 90° (Figure 16), but again they show greater dispersion about that mean. The large lesion group shows mean angle of rotation close to +/-90° during the cue rotation sessions. Categorising place field responses revealed no difference between the two lesion groups.

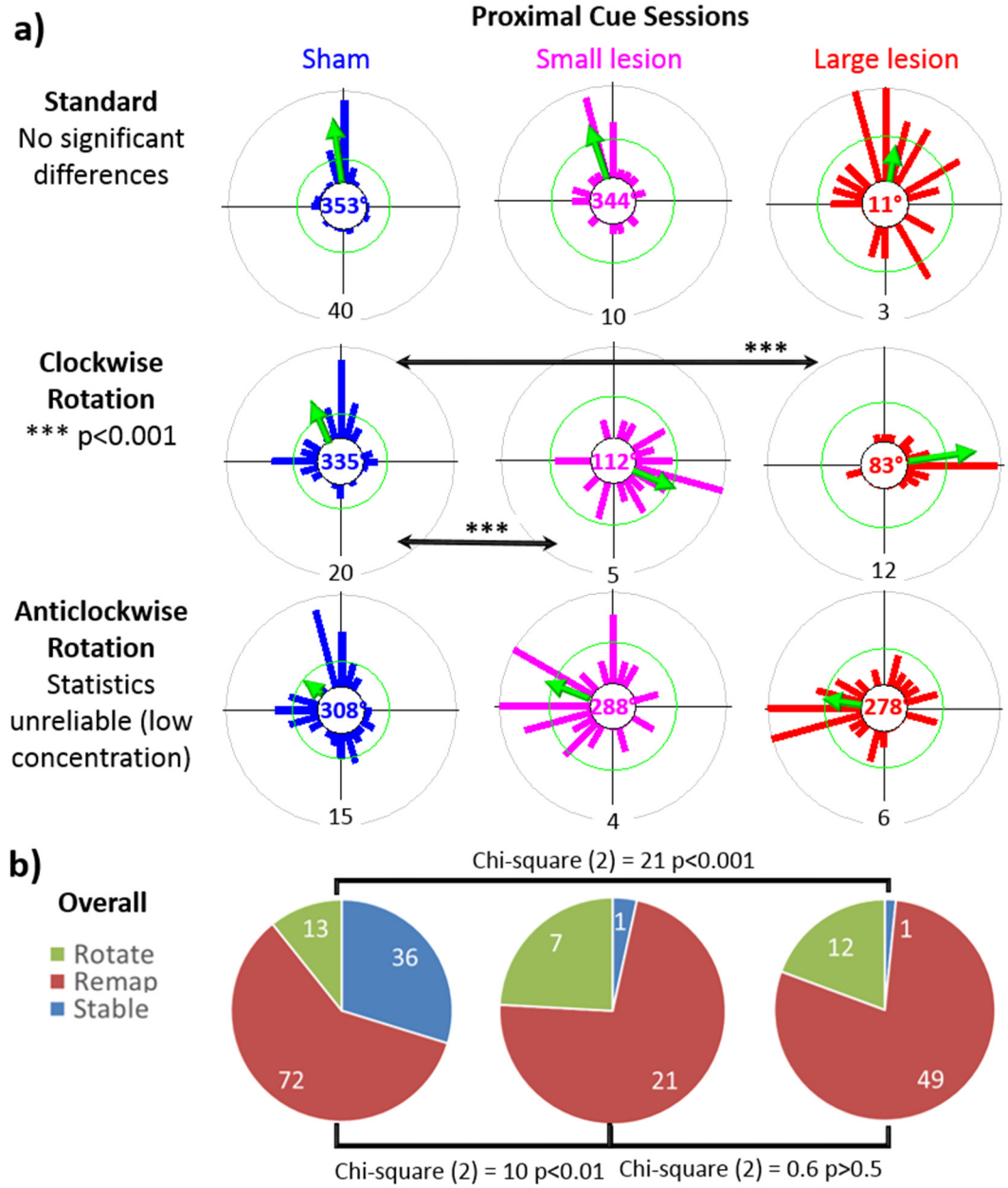


Figure 16: a) Proximal cue rotation: Circular histograms displaying frequency of cells with each angle of best correlation between standard 1 and 2 (row 1), standard 2 and rotation (row 2) and rotation and standard 3 (row 3). The number in the centre of each plot displays the mean angle (μ), which is also displayed as a green arrow. The length of the arrow (r) is a measure of how tightly the distribution is clustered around this mean. The green circle displays the Rayleigh's critical value for the data. b) Pie chart showing the overall behaviour of the cells in each experimental group categorised as either showing remapping, stability relative to the room, or stability relative to the cues across 3 sessions.

4.4 Discussion

4.4.1 The effects of MEC lesions on the firing properties of CA1 place cells and the anchoring of their place fields to distal and proximal cues

Consistent with all previous studies (Miller and Best 1980, Brun *et al.* 2008, Van Cauter *et al.* 2008, Hales *et al.* 2014), spatially modulated place cell activity was still present in animals with MEC lesions. However place cells recorded here also showed reduced spatial precision which matches some previous studies (Brun *et al.* 2008, Hales *et al.* 2014, Ormond and McNaughton 2015). Spatial information content and sparsity, both of which are measures of the spatial precision of place cell activity, showed changes consistent with a decrease in spatial precision.

Consistent with previous studies, there was a decrease in peak firing rate (Van Cauter *et al.* 2008, Hales *et al.* 2014), but there was no consistent effect on average overall firing rate. This may be explained by the increase in sparsity, as it is possible that increased out of field firing negated the lower peak firing rate resulting in little change to the overall average firing rate. However Van Cauter *et al.* (2008) saw a decrease in all measures of firing rate, so this result is not entirely consistent with previous research. It does however match the results of Ormond and McNaughton, who found a decrease in peak firing rate but no significant effect overall, which was also explained by the increase in out of field firing. Conversely Hales *et al.* (2014) suggested that the reduced firing rate could be explained by an increased number of low-firing cells with a peak firing rate less than 1 Hz. However in our data even when cells firing with a mean peak firing rate less than 1 Hz were excluded, there was still a significant decrease in peak firing rate suggesting that MEC lesions reduce the peak firing rate of active cells rather than just increasing the proportion of cells which are not active. There was not a consistent effect upon stability between sessions. In the distal cue environment there was no effect on stability compared to the sham group, but in the proximal cue environment place cells in the lesion group showed reduced stability compared to shams. Previous studies have seen reduced stability between session and

	Manipulation	Spatial Information	Field size	Stability	Peak firing rate	Other observations
Miller & Best 1980	EC lesion (mainly MEC)	↓ *				Place fields not anchored to extramaze cues *robustness rather than SI
Brun *et al.* 2007	MEC LIII lesion	↓	↑	↓	–	
Van Cauter *et al.* 2008	MEC lesion	–	↓	↓	↓	Decrease in following rotating object cues. Increased remapping following cue removal
Hales *et al.* 2014,	MEC lesion	↓ *	↑	↓	↓	*due to drifting place fields rather than wider place fields
Ormond *et al.* 2015	Dorsal or Ventral MEC inactivation	↓	↑		↓	
This study	MEC lesion	↓	*	D– ** P↓	↓	* not tested, although sparsity and visualising rate maps suggests field size increases ** stability varies depending upon the cues available

Table 9: Comparison of these results with previous studies (↓ reduced, ↑ increased, – unchanged, D distal cues, P proximal cues)

it is not clear why this was not observed in the distal cue sessions. Table 9 summarises the results described here and allows comparison with previous results.

Interestingly, place cells recorded from MEC lesion animals in this experiment did not rotate their place fields with distal cues, particularly in the animals with large lesions. Failure to rotate with distal cues suggests that distal visual information, which would provide polarising directional information, is not influencing hippocampal place cells. This result suggests that distal cue information reaches the hippocampus via the MEC. This result matches the data from Miller and Best (1980), and explains the results of several behavioural studies which have suggested deficits in navigating using distal cues following MEC lesions (Parron *et al.* 2004, Hales *et al.* 2014). This experiment

also found that although place fields in lesion animal were not anchored to distal cues, they could be anchored to proximal cues and in fact showed stronger anchoring to proximal cues than cells from the sham animals. This suggests that proximal cue information reaches the hippocampus via a different pathway. Collectively, these two results suggest that the reason that place cell activity still shows spatial modulation following MEC lesions may be due to the fact that in most environments, location can be determined relative to a mix of both distal and proximal cues. In the absence of the MEC input of distal cue information, proximal cue information might be enough to stabilise place fields. This is also consistent with previous behavioural studies which have shown that MEC lesioned rats showed impaired navigation in the water maze using distal cues but were not impaired on navigation using proximal cues (Parron *et al.* 2004). In contrast, parietal cortex lesions impaired proximal cue use but not distal cue use, while hippocampal lesions impaired the use of both types of cue (Save and Poucet 2000), suggesting that there are different pathways involved in the processing of the different types of cue. It therefore seems likely that lesions of the MEC disrupt the pathway which brings distal cue information to the hippocampus while sparing the pathway bringing proximal cue information to the hippocampus.

These results also raise the possibility that the previous and current findings of decreased place field precision following MEC lesions may be the result of the place cells being deprived of information from distal cues. The reason for the decreased field precision may be that animals are unable to use distal cues and so must rely on any remaining proximal cues, which may be very minimal or unreliable. Distal cue information is generally more informative for determining orientation because motion parallax is greater for proximal cues. Consistent with this, one of the two previous experiments that found decreased place field precision following MEC lesions (Brun *et al.* 2008) used predominantly distal cues, reducing the availability of proximal cues to imperfections in the apparatus or odour cues generated by the animals themselves. The other study used a cue card on the wall of the environment (Hales *et al.* 2014). Cue cards may be treated as distal cues (Scaplen *et al.* 2014). Since cue rotation was not performed in either study it is not possible to ascertain whether the cells were using

the cues for orientation but the data here would suggest that they were not. Conversely, the previous study that did not find a reduction in place field precision used object cues rather than distal cues (Van Cauter *et al.* 2008).

Alternatively, the decrease in place field precision may imply that the MEC has a role in refining place field precision which is separate from its role in determining place field location relative to distal cues. If the only reason for the reduced spatial precision of place fields following MEC lesions is that they can no longer use distal cues, it might be expected that spatial precision would increase in the proximal cue environment. This did not occur, which suggests that the reduction in place field precision following MEC lesions may be a separate effect rather than another facet of the effect on distal cue processing. However there is strong evidence that the MEC lesion animals were relying on some other type of cue at least in the distal cue sessions, since a high proportion of cells showed stability across sessions even when the distal cues rotated. This unknown cue (possibly local odour cues) may provide good spatial information and explain why there were good levels of stability in the distal cue sessions, and why there was no difference is spatial precision between the distal and proximal sessions in the lesion animals.

4.4.2 Comparison with previous studies

There are several methodological differences between the experiment described here and the previous experiments. Firstly, only Hales *et al.* (2014) are describing the effect of complete but restricted to MEC lesions on CA1 place cell activity. Both Miller and Best (1980) and Van Cauter *et al.* (2008) aimed to hit the entire entorhinal cortex rather than just the medial entorhinal cortex. Although Miller & Best claim that their lesions include the LEC as well as MEC, comparing their lesion schematics with the Paxinos and Watson rat brain atlas (1998) reveals sparing of the more anterior or lateral LEC. Similarly, the lesions performed by Van Cauter *et al.* (2008) are relatively complete in the MEC but show sparing in the LEC. Therefore it is entirely possible that in both of these studies, the lesions are relatively complete in the MEC but do not cover enough of the LEC to have an effect. Brun *et al.* (2008) used a neurotoxin to selectively lesion only layer III of the MEC. Lesions of Layer III MEC would remove the direct input

from the MEC to the CA1 cells recorded in the study (Brun *et al.* 2008), however they would spare the indirect input via DG and CA3 which originates in Layer II MEC. CA3 cells are known to show cue rotation following distal cues (Lee *et al.* 2004) suggesting that the layer II MEC input to the dentate gyrus and CA3 carries distal cue information. Interestingly, despite the fact that these lesions only removed the direct input to CA1 place cells, but spared the indirect input, the effects on place cell precision and stability were similar to the effects seen in this experiment and other complete MEC lesion studies.

The lesions described in this thesis were not well restricted to the MEC. In two animals there was substantial damage to the ventral hippocampus and ventral presubiculum, while in the other two animals, although there was no damage outside of the MEC, there was also much more sparing within the MEC. It is therefore not possible to establish whether the differences between the small lesion and large lesion groups are due to the effects of ventral hippocampus or ventral presubiculum lesions or whether they are due to the effect of complete MEC lesions.

Place cells in the ventral hippocampus have larger place fields than in dorsal hippocampus (Royer *et al.* 2010, Keinath *et al.* 2014), and lesion studies generally show no deficits or much weaker deficits in spatial navigation tasks following ventral hippocampus lesions (Ferbinteanu and McDonald 2000, Pothuizen *et al.* 2004, Zhang *et al.* 2004,). The ventral hippocampus seems to have roles in anxiety (Bannerman *et al.* 2004), retrieval of a spatial memory learned with an intact hippocampus (Loureiro *et al. 2012),* and generalization across contexts (de Hoz and Martin 2014), but none of those roles seem likely to affect the ability to process distal cues. It is therefore unlikely that the effects described here are the result of the damage to the ventral hippocampus. The presubiculum is a key part of the head-direction circuit (Taube *et al.* 1990) and projects strongly to the medial entorhinal cortex (van Haeften *et al.* 1997). It is therefore possible that the reason for the effects seen in this experiment are due to the damage to the presubiculum. However the lesions only included the ventral presubiculum and did not spread to the dorsal parts of the presubiculum (also known as the postsubiculum). The presubiculum projects to the medial entorhinal cortex in a

topographic manner with ventral presubiculum projecting to ventral MEC and dorsal presubiculum (postsubiculum) projecting to the dorsal MEC which is more spatially precise (van Haeften *et al.* 1997), suggesting that the dorsal presubiculum may provide more precise orientation information. In addition, most of the studies on head direction activity have focussed on the role of the postsubiculum rather than the ventral presubiculum. Lesions of the postsubiculum which spared the ventral presubiculum caused a reduction in place cell anchoring to distal cues (Calton *et al.* 2003). This suggests that the postsubiculum may have a more important role in processing head-direction information based on distal cues than the ventral presubiculum. If this is the case, then it would suggest that the results observed here are caused by the MEC lesions rather than the presubiculum damage. However it is also possible that damage to the ventral presubiculum may disrupt activity in the postsubiculum and thereby impair the use of distal cues. Further experiments in which the MEC is lesioned without associated damage to the ventral presubiculum are needed to confirm that the effects described here are due to the MEC damage rather than the ventral presubiculum damage.

Secondly, the cues differed between experiments. Of the previous experiments, only Van Cauter *et al.* (2008) performed cue rotation experiments, however although they saw a decrease in the proportion of cells showing rotation, they did not find the complete deficit seen here. One possible reason for this is the types of cues they used. Distal cues and cue cards can only be perceived from one side whereas proximal object cues can be seen from multiple angles and, if not placed at the very edge of an environment, can also be circumnavigated. The cues used by Van Cauter *et al.* were object cues similar to those used in the proximal sessions here but were placed at the very edge of the environment. Since there appears to be a difference between cues which are at the edge (touching the walls) of an environment, and cues which do not touch the edge of the environment (Scaplen *et al.* 2014) it is not clear whether their cues would be processed by the distal cue pathway or the object cue pathway (or perhaps both). The effect of MEC lesions in their environment is somewhere in between the results seen here in the distal and proximal cue sessions described here,

with increased remapping compared to control animals, but still some evidence of anchoring to cues, with 49% of cells in the lesion group showing place fields which rotated with the cues. Object cues which are not touching the wall but which are close to the wall can exert an influence on place field orientation in a cylinder but object cues placed near the centre do not (Cressant *et al.* 1997). Since the object cues used in the experiment described in this chapter are near the edge of the environment rather than actually touching the edge, they are unequivocally proximal cues, but are not so close to the centre of the environment that they would be expected to exert no control over the orientation of place cells. With this cue environment, there was no evidence of anchoring to the distal cues but very good anchoring to the proximal cues in the lesion group. The intermediate result from Van Cauter *et al.* may suggest that with distally placed object cues, both the distal cue pathway and proximal cue pathway may be able to support cue anchored place fields resulting in a lesser deficit compared to shams following lesions to the distal cue pathway.

Another important difference is the species used in the experiments. Mice show reduced place field stability over time compared with rats, particularly when attention to the environment is not necessary for solving a task (Kentros *et al.* 2004). As the previous experiments used rats rather than mice it is difficult to compare the amount of remapping seen previously (for example in Van Cauter *et al.* 2008) and this experiment since there is much more remapping in the sham mice studied here than has been seen previously in control rats. Miller and Best (1980) did not perform cue rotation experiments, but as mentioned previously, their data supports the idea that in rats also MEC lesions cause a change from the use of extramaze cues to intramaze cues in determining place field location. As behavioural experiments in rats with MEC lesions have also shown deficits in navigation using distal but not proximal cues (Parron *et al.* 2004), it is likely that the results on cue rotation described here would be similar in rats, but that the results on extent of remapping between sessions may be somewhat different, particularly when comparing the proportion of cells from the sham group which show remapping between sessions.

The result that the place cells from the sham animals did not rotate with the proximal cues is consistent with the data from previous studies investigating the extent to which place fields are anchored to objects (Yoganarashimha *et al.* 2006, Cressant *et al.* 1997, Scaplen *et al.*2014). In the former study, a greater proportion of CA1 place cells rotated with distal cues than rotated with proximal cues when the two cue types were put into conflict with each other (Yoganarashimha *et al.* 2006), although contradicting this, Renaudineau *et al.* (2007) found that a proportion of cells followed distal cues and a proportion followed proximal cues, which in this case were objects placed at the extreme edge of the environment. Scaplen *et al.* (2014) used purely visual cues projected onto the floor or walls rather than physical objects, and found that place fields would rotate with cues on the wall of an environment, or large cues on the floor of an environment (which touched the wall), but would not rotate with smaller 'object' cues projected onto the floor located 4 cm from the wall (Scaplen *et al.* 2014). In animals who never experienced wall based cues, a smaller proportion of place fields would rotate with the 'object' cues. Similarly in our data few place cells from the control animals showed rotation with the objects. However many more cells from the lesion animals, who might not have been able to perceive or use the distal cues in previous sessions, did show field rotation with the object cues.

It is unclear why a high proportion of place fields from the sham animals showed stability throughout the proximal cue sessions. Although complete removal of other cues was attempted, it is possible that these animals were using other distal cues in the environment that had not been well controlled and hence were detecting the proximal cue rotations. If this were the case it might result in the almost complete lack of place field rotation which was observed here.

Another result that is difficult to explain is the place field stability between the distal cue rotation sessions for MEC lesion animals. Since the floor of the environment remained in the same position in all sessions one possible explanation for the stable cells are local olfactory cues that were not completely removed by cleaning between sessions. In addition it is possible that there were polarising olfactory, auditory or lighting cues which may not have been completely eliminated. Also, although we

covered the animals up in the home cage, and rotated them a small amount before placing them back in the environment from a different direction, it is possible, although unlikely, that they were able to maintain a sense of direction using idiothetic cues. This sense of direction may have been strong enough to provide some stability to place fields between sessions in the absence of any conflicting cues, which may have been the case if the lesion animals were unable to process the directional information from the distal cues.

4.4.3 Implications of this result

The finding that the MEC provides distal cue information to the hippocampus but seems not to be involved or at least not to be necessary for proximal cue based navigation has implications for our understanding of the role of the MEC in navigation. Previously, due to the repeating nature of grid cells, it has been suggested that the MEC might play a role in path integration. However, it is possible that rather than being involved in path integration, grid cells may actually be involved in maintaining the distal reference frame. However this does not fit particularly well with the observation that the grid cell lattice 'fragments' when in an environment with repeating local boundaries which suggests that grid field position is more strongly modulated by the local boundaries than by a global representation of the environment (Derdikman *et al.* 2009). Another role for the grid cell input might be to refine the spatial precision of place cells, particularly in areas of the environment which are far from boundaries. This would be consistent with the effects described here that MEC lesions reduce place field precision. Medial septum inactivation disrupts grid cell firing without removing either head-direction coding or border cell coding within the MEC (Brandon et al 2011), and this manipulation has been used to show that the grid cell input is not necessary for place field generation in a new environment (Brandon et al 2014). It might be interesting to check whether medial septum inactivation has a similar effect to the results described here.

Head direction cells are potentially a more convincing source of directional information based on distal cues. Head direction cells are capable of using visual cues as well as vestibular and proprioceptive inputs to generate a representation of the

animal's orientation. It has been proposed that visual distal cue information is integrated into the head direction circuit at the postsubiculum (Goodridge and Taube 1997) which projects to the medial entorhinal cortex. In support of this hypothesis, lesions of the postsubiculum prevent head direction cells in the anterior thalamic nucleus from being controlled by distal landmarks (Goodridge and Taube 1997). Additionally, it has been observed that following postsubiculum lesions, place fields no longer follow distal cues (Calton *et al.* 2003). Rotation of a large visual distal cue did not cause rotation of place fields, although its removal did cause remapping. This again suggests that the integration of distal visual cues into a representation of orientation occurs at the level of the postsubiculum. Since the postsubiculum does not project directly to the hippocampus but projects strongly to the MEC (Swanson and Cowan 1977), it is likely that this is the route by which this information reaches hippocampal place cells.

There are also models of place field formation which rely on the input from boundary vector cells (BVCs) (O'Keefe and Burgess 1996, Hartley *et al.* 2000). BVCs are found in both the subiculum and the MEC and both populations may project to hippocampal place cells (Sun *et al.* 2014, Zhang *et al.* 2013). In computer models, the summation of inputs from multiple border cells has been found to result in activity similar to a place field (Hartley *et al.* 2000). Since boundary vector cells also have a strong directional component it is possible that these cells are also carrying distal cue information into the hippocampus. Since our lesions did not include the subiculum, it is possible that the subiculum input to CA1 is supporting place cell activity. However if this is the case, it suggests that the orientations of BVCs in the subiculum are not anchored to distal cues, at least in the absence of the input from the MEC.

It seems most likely, given the diverse input from the MEC to hippocampus, that all the spatial cell types found in the MEC show some degree of directional tuning to distal cues, probably because of inputs from the postsubicular head direction cells. Removal of the MEC prevents this information from reaching the hippocampus either directly or possibly indirectly via the subiculum.

This result also raises the question of where the proximal cue information is coming from. The most likely possibility is the input from the LEC. As mentioned previously, activity in LEC cells has been shown to correlate to the proximal reference frame in experiments which put the proximal and distal reference frames into conflict (Neunuebel *et al.* 2013). LEC cells show spatial tuning similar to that seen in place cells when in environments containing objects, and in addition, if a familiar object is moved, some LEC cells appear to represent the object's previous location even in the absence of the object itself (Deshmukh and Knierim 2011). Therefore there is evidence that LEC cells represent more than just the objects themselves, but also integrate them with their locations within an environment. This integrated representation of objects and locations might be enough to allow the cue rotations seen in this experiment, particularly for locations near to one of the objects. This hypothesis could be tested by repeating the current cue rotation experiments in animals with LEC lesions. If proximal cue information is entering the hippocampus via the LEC it would be expected that LEC lesions would not impair the anchoring of place fields to distal cues, but would reduce the anchoring of place fields to proximal cues. Since very little proximal cue anchoring was seen in the sham group in this experiment, it might be necessary to choose a protocol in which greater anchoring to proximal cues is typically seen, for example removing all distal cue information by performing the experiment in a cylinder (Zugaro et al 2001), or using a circular track on which animals are trained to run in one particular direction (Lee *et al.* 2004).

Another possible brain region that has been implicated in the processing of proximal cues for navigation, is the parietal cortex. Lesions of the parietal cortex impaired navigation in a proximal cue version of the water maze reference memory task but did not impair the use of distal cues (Save and Poucet 2000). However, the parietal cortex has no direct inputs to the hippocampus which makes it unlikely as a source of directional information to place cells.

This experiment reveals that the information from distal cues which allows place cells to orient themselves within an environment, enters the hippocampus from the MEC. It is hypothesised that without this information entering the hippocampus, navigation

using distal cues should be impaired. This was not tested here but could be tested by performing watermaze experiments using a variable start location to promote allocentric navigation and restricting the cues available to the animal to either distal or proximal cues as well as using the various strategies for masking other auditory or light-based polarizing cues described here. It would then be possible to test whether any navigation was based upon the controlled cues, by rotating them and performing a probe trial to measure whether the animal spent a significant proportion of time in the original platform location, or whether their area of dwelling had rotated around the maze consistently with the cues.

5 Conclusions and future directions

The aim of this thesis was to investigate three different inputs to CA1 place cells to explore their possible contributions to place cell activity and behaviour. The three major inputs investigated were CA3, which is thought to have a role in spatial learning and memory, the nucleus reuniens, which is thought to have a role in strategy selection and working memory, and the MEC, which is thought to contribute current spatial information.

5.1 Parallel pathways for the generation of trajectory-dependent activity in CA1

Trajectory-dependent activity has been well studied in CA1 and usually appears when a task is learned in which animals pass through the same location on multiple different trajectories. This activity has been observed elsewhere in the brain, but there were conflicting results on whether it occurred in CA3 (Bahar and Shapiro 2012, Ito *et al.* 2015). It was hypothesised that although trajectory-dependent activity is not seen in CA3 during the hippocampus-independent T-maze alternation task, CA3 place cell activity would be modulated by trajectory during performance of a hippocampus-dependent navigation task. As was predicted, CA3 place cells showed trajectory-dependent activity in the experiment described in Chapter 2. In all respects this seemed similar to that seen in CA1 in this task. It developed as the task was learned, and occurred in approximately 40% of active CA3 place cells as has previously been seen in CA1 place cells. This result confirms previous reports of trajectory-dependent activity in CA3 (Bahar and Shapiro 2012) and adds support to the idea that it occurs in spatial tasks that require the hippocampus. However it contradicts the previous result that CA3 place cells show much less trajectory-dependent activity than CA1 cells during the T-maze alternation. This could be explained since the task is not hippocampus-dependent (Ainge *et al.* 2007a). More testing in other tasks is therefore necessary to confirm whether CA3 trajectory-dependent activity is consistently reduced in hippocampus-independent maze tasks but consistently present in hippocampus-dependent tasks.

The primary result in Chapter 3 is that the nucleus reuniens is not required for acquisition or performance of the double-Y-maze task. In a previous study Ito *et al.* (2015) found that trajectory-dependent activity was reduced in CA1 following lesions or temporary inactivation of the reuniens. This was shown in the T-maze alternation task, in which CA3 trajectory-dependent activity is much weaker. Reuniens inactivation had no effect on accuracy, but the task does not require the hippocampus and therefore the input from reuniens to hippocampus is presumably not necessary for performance. We tested whether reuniens lesions would disrupt the ability to perform the hippocampus-dependent double-Y-maze task in which trajectory-dependent activity is seen, and which develops in parallel with task acquisition in both CA3 and CA1.

If the reuniens input is the only source of trajectory-dependent activity in the hippocampus then it would be predicted that nucleus reuniens lesions would cause deficits on the double-Y maze task, particularly during reward switches when trajectory-dependent activity could potentially convey information about the current goal from areas in the prefrontal cortex. Alternatively, if alternative inputs to CA1 can provide this activity then reuniens inputs may have little effect on performance. We found no evidence of an impairment on task acquisition or performance following reuniens lesions, leading to one of two possible conclusions; either the nucleus reuniens input is not necessary for trajectory-dependent activity in the double-Y-maze, or trajectory-dependent activity is not necessary for successful task performance. Taken together with the results described in Chapter 2 in which trajectory-dependent activity was observed in CA3 on this task, it seems most likely that reuniens lesions do not reduce trajectory-dependent activity in CA1 in this task. Further experiments in which place cells are recorded during reuniens lesions or silencing during acquisition of the task would determine which of these possibilities is correct. My prediction, based on the previously conflicting results on the presence of trajectory-dependent activity in CA3 and the current finding of CA3 trajectory-dependent activity in this task, is that the nucleus reuniens is not required for the emergence of trajectory-dependent activity in CA1 in this task. If this is the case then lesions of the nucleus

reuniens should have no effect on trajectory-dependent activity on the double-Y-maze. This would suggest that the CA3 inputs to CA1 drive trajectory-dependent activity in CA1 in this task, however it does not rule out the possibility that it is dependent upon inputs from another structure such as the MEC. This could potentially be tested by selectively silencing CA3 and recording from CA1 during performance of the double-Y-maze task. This should not block basic place cell activity in CA1 (Brun *et al.* 2002), but would block trajectory-dependent activity if it is derived from the CA3 inputs. This experiment would have the potential both to determine whether trajectory-dependent activity in CA1 is dependent upon different inputs in different tasks, and to increase our understanding of the functional role of trajectory-dependent activity in task acquisition and performance of hippocampus-dependent tasks.

The cellular mechanism by which trajectory-dependent place cell activity arises has also not being investigated fully. It seems likely, based on the electrophysiological data, that the nucleus reuniens inputs induce trajectory-dependent activity by modulating the excitability of pyramidal neurons, causing rate-remapping of already established place fields. Since this trajectory-modulation disappears immediately with removal of the reuniens input it appears that it is an ongoing modulation rather than causing changes in CA1 capable of producing trajectory modulation in the absence of an input from the nucleus reuniens. In the case of reuniens inputs this fits in with its suggested role in working memory. However in the case of CA3 inputs, it is possible that plasticity either within the CA3 recurrent circuit or plasticity of the projection to CA1 might be critical for the development of trajectory-dependent activity and is another avenue for future investigation.

5.2 The nucleus reuniens and navigational strategy selection

The nucleus reuniens is the most direct route of information flow from the mPFC to the hippocampus. Following the result described above that this input is not necessary for acquisition of the double-Y-maze serial reversal task, several additional experiments explored the role of the nucleus reuniens in tasks that differentiated between allocentric and egocentric navigation. The watermaze deficit suggests that the reuniens is involved in the selection or application of an allocentric navigational

strategy. However this is called into question by the fact that no deficit was found in learning a place task on the plus maze. Conversely, reuniens lesions were found to enhance the speed at which an egocentric task was learned on the plus maze. A possible explanation is that the prefrontal cortex to hippocampus connection is involved in spatial problem solving particularly when this is allocentric. When presented with a change in task on the plus maze the absence of this connection may allow an egocentric strategy to be identified as successful and adopted more quickly because of a lack of interference from an allocentric strategy. There is some support from previous literature for the idea that the reuniens is involved in switching between egocentric and allocentric strategies, since nucleus reuniens lesions cause a deficit in switching to an allocentric strategy following failure of an egocentric strategy on the double H maze (Cholvin *et al.* 2013). However again this should produce deficits in learning the place task on the plus maze, which did not occur in our experiment suggesting that the reuniens is not always necessary for switching to an allocentric navigation strategy. Therefore further research is needed to reconcile these results and determine the precise role of the reuniens in allocentric navigation.

5.3 MEC lesions disconnect place cells from distal cues

The results described in Chapter 4 indicate that the MEC inputs provide information about distal cues to hippocampal place cells. Without their input, place cells rely on proximal cues to orient their place fields within an environment. In addition, MEC lesions also caused an increase in place field size and decrease in spatial information content. These results are consistent with previous results that place cells still show place fields following MEC lesions, however in addition these results suggest that the MEC is necessary specifically for conveying distal cue information to the hippocampus. These results are similar to the effects seen following postsubiculum lesions (Calton *et al.* 2003); lesions of the postsubiculum and to a lesser extent the ATN caused place cells to lose stability relative to a cue-card. Since the postsubiculum projects strongly to the MEC (van Haeften *et al.* 1997), and the main cell types within the MEC show rotation with distal cues (Solstad *et al.* 2008, Neuneubel *et al.* 2013), it makes sense that this information reaches the hippocampus via the MEC. It is

interesting that orientation relative to proximal cues appears to be unimpaired following MEC lesions, suggesting an alternate pathway supports proximal cue processing. Since experiments have shown that LEC neurons show spatially modulated activity near to objects or in environments with many objects (Deshmukh and Knierim 2011), and that their activity shows rotation with proximal cues (floor textures) (Neunuebel *et al.*.2013), this is suggested to be the source of proximal cue information to the hippocampus (Knierim *et al.* 2014). The fact that place cells in intact animals do not rotate with the proximal cues suggests that the proximal cue input is usually less dominant and that place cells predominantly rely on the MEC inputs when they are available, but that when this input is not available place cells are able to form stable fields using proximal cues.

5.4 Parallel and compensatory inputs to the hippocampus

John O'Keefe in his 1979 review described a place cell as "a cell which constructs the notion of a place in an environment by connecting together several multisensory inputs each of which can be perceived when the animal is in a particular part of an environment." The results described here suggest that these multisensory inputs come from different regions depending on the nature of the spatial information they contain. The results suggest place cell activity can be driven by multiple parallel pathways into the hippocampus. This allows place cell activity to be stabilized by whatever cues are available. In normal life this would allow flexible navigation in response to different environments. In this and other studies, it is manifested in the surprising resilience of place cell activity to selective removal of inputs to the place cell population, allowing place fields to appear superficially unaffected no matter which of the main inputs are removed. The results described in this thesis suggest that the inputs from CA3 may carry trajectory information to CA1 and that the trajectory-modulated input from the nucleus reuniens is not necessary for acquisition of a trajectory based task that requires the hippocampus. It also suggests that the MEC provides the hippocampus with distal cue information that cannot be provided by the inputs from the LEC, but confirms that LEC (or possibly other) inputs are sufficient to drive place cell activity in CA1, and to allow stabilisation with respect to proximal but not distal cues.

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